Insulin Releases Glut4 from Static Storage Compartments into Cycling Endosomes and Increases the Rate Constant for Glut4 Exocytosis

Joseph M. Muretta, Irina Romenskaia, and Cynthia Corley Mastick

From the Department of Biochemistry and Molecular Biology, University of Nevada, Reno, Nevada 89557

In adipocytes, insulin triggers the redistribution of Glut4 from intracellular compartments to the plasma membrane. Two models have been proposed to explain the effect of insulin on Glut4 localization. In the first, termed dynamic exchange, Glut4 continually cycles between the plasma membrane and intracellular compartments in basal cells, and the major effect of insulin is through changes in the exocytic and endocytic rate constants, $k_{\text{ex}}$ and $k_{\text{en}}$. In the second model, termed static retention, Glut4 is packaged in specialized storage vesicles (GSVs) in basal cells and does not traffic through the plasma membrane or endosomes. Insulin triggers GSV exocytosis, increasing the amount of Glut4 in the actively cycling pool. Using a flow cytometry-based assay, we found that Glut4 is regulated by both static and dynamic retention mechanisms. In basal cells, 75–80% of the Glut4 is packaged in noncycling GSVs. Insulin increased the amount of Glut4 in the actively cycling pool 4–5-fold. Insulin increased the dynamic retention mechanisms. In basal cells, 75–80% of the Glut4 is packaged in specialized storage vesicles (GSVs) in basal cells and does not traffic through the plasma membrane or endosomes. Insulin triggers GSV exocytosis, increasing the amount of Glut4 in the actively cycling pool. Using a flow cytometry-based assay, we found that Glut4 is regulated by both static and dynamic retention mechanisms. In basal cells, 75–80% of the Glut4 is packaged in noncycling GSVs. Insulin increased the amount of Glut4 in the actively cycling pool 4–5-fold. Insulin also increased $k_{\text{ex}}$ in the cycling pool 3-fold. After insulin withdrawal, Glut4 is rapidly cleared from the plasma membrane ($t_{1/2}$ of 20 min) by rapid adjustments in $k_{\text{ex}}$ and $k_{\text{en}}$ and recycled into static compartments. Complete recovery of the static pool required more than 3 h, however. We conclude that in fully differentiated confluent adipocytes, both the dynamic and static retention mechanisms are important for the regulation of plasma membrane Glut4 content. However, cell culture conditions affect Glut4 trafficking. For example, replating after differentiation inhibited the static retention of Glut4, which may explain differences in previous reports.

The rate of glucose uptake in muscle and adipose tissue is limited by the total number of facilitative glucose transport proteins inserted into the plasma membrane (1, 2). Insulin regulates glucose uptake in fat and muscle by controlling the subcellular distribution of glucose transporters, predominantly the Glut4 isoform. In the absence of insulin, Glut4 is largely excluded from the plasma membrane and is retained within endosomes, the trans-Golgi network, and tubular-vesicular compartments termed Glut4 storage vesicles (GSVs) (1, 2). Insulin increases cell surface Glut4 through an increase in the total amount of Glut4 in the actively cycling pool as well as through an increase in $k_{\text{ex}}$. Early kinetic work used photoactivated glucose analogs to label Glut4 at the cell surface (6, 7). Glut4 labeled at the cell surface at 4 °C redistributed to intracellular compartments when cells were warmed to 37 °C, under both basal and insulin-stimulated conditions. Insulin increased $k_{\text{ex}}$ and decreased $k_{\text{en}}$.

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To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Mailstop 330, University of Nevada, Reno, NV 89557. Tel.: 775-784-1155; Fax: 775-784-1419; E-mail: cmastick@unr.edu.
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These observations suggested that the distribution of Glut4 was controlled through dynamic exchange and that the effect of insulin on Glut4 localization was due to effects on the rate constants for exocytosis and endocytosis. In these studies, it was explicitly assumed that all of the Glut4 actively cycled between the plasma membrane and intracellular compartments in both basal and insulin-stimulated cells. The size of the actively cycling pool was not directly determined.

More recently, exo facial epitope-tagged Glut4 reporter constructs have been used to study Glut4 traffic in live cells (3, 4, 8–11). Unlike endogenous Glut4, these reporters can be labeled with antibody when they are inserted into the plasma membrane. When expressed at low levels, the reporters traffic with the endogenous Glut4 (3, 8, 12). Both the rate constant of exocytosis and the size of the total cycling pool can be determined from the kinetics of antibody binding/uptake during continuous labeling at 37°C. Using this method, Martin et al. (3) concluded that the distribution of Glut4 in 3T3-L1 adipocytes was controlled by dynamic exchange. In their experiments, ~90% of the total Glut4 was cycling in both basal and insulin-stimulated cells. The major effect of insulin was to increase \( k_{ex} \) by 7–10-fold. Insulin also decreased \( k_{en} \) by 70%. However, using the same cells, the same reporter constructs, and the same antibodies, Govers et al. (4, 5) found that the distribution of Glut4 was controlled by static retention. The major effect of insulin was to increase the size of the actively cycling pool by ~7-fold (from 10% of the total to 70% of the total Glut4 in the cell). Insulin also increased \( k_{ex} \) by 2-fold.

These two models suggest very different mechanisms regulating Glut4 traffic. To determine the physiological relevance of these observations, it is important to determine how differences in the experimental methods used in the two studies influence Glut4 traffic. Previous reports using anti-HA uptake to measure the kinetics of Glut4 traffic have either relied on single cell analysis (3) or whole plate assays (4). We developed a flow cytometry based assay to analyze the kinetics of Glut4 exocytosis (we had previously used flow cytometry to measure the kinetics of endocytic trafficking of transferrin and \( \alpha-2\)-macroglobulin) (13–15). Flow cytometry combines the ability of microscopy to analyze individual cells with the ability of the whole plate assay to analyze large numbers of cells in each sample. We evaluated the effects of methodological differences between the studies of Martin et al. (3) and Govers et al. (4) on Glut4 trafficking in differentiated 3T3-L1 cells. Specifically, we tested the effect of replating quiescent, confluent differentiated cells, as is commonly done in electroporation and/or microscopy experiments using the 3T3-L1 cell culture model.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**3T3-L1 fibroblasts (CCL 92.1; American Type Culture Collection) were maintained in DMEM (high glucose, with 2 mm l-glutamine, 50 units/ml penicillin, and 50 \( \mu \)g/ml streptomycin) supplemented with 10% calf serum (CS; HyClone). For experiments, cells were plated into individual wells of a 96-well plate (0.12 \( \times \) \( 10^5 \) cells/well). At confluence (3 days after plating), the cells were switched to DMEM supplemented with 10% fetal bovine serum (FBS; HyClone; characterized) and 5 \( \mu \)M Troglitazone (Calbiochem). 1 day postconfluence (day 0), the cells were induced to differentiate into adipocytes essentially as described (16). Briefly, cells were incubated for 3 days in differentiation medium (DMEM, 10% FBS, 5 \( \mu \)M Troglitazone, dexamethasone, isomethylbutylxanthine, and insulin), 3 days in insulin medium (DMEM, 10% FBS, Troglitazone, and insulin), and then 4–5 days in DMEM, 10% FBS. Cells were used 8–11 days after the initiation of differentiation. 3T3-L1 fibroblasts were used 2–3 days postconfluence. To test the effect of confluence on Glut4 traffic, differentiated 3T3-L1 cells were lifted from the culture plates by collagenase digestion and replated at lower density. Briefly, cells were differentiated in 96-well plates as described. Upon removal of insulin-containing medium, cells were incubated with collagenase (1 \( \mu \)g/\( \mu \)l in PBS plus 1% BSA, 20 \( \mu \)l/well, 5 min, 37°C), fresh culture medium (DMEM, 10% FBS) was added to each well (200 \( \mu \)l/well), and cells were suspended by gentle pipetting. 100 \( \mu \)l from each well was transferred to a new 96-well culture plate. Cells were allowed to settle for 2 h. After settling, cells were refed with 120 \( \mu \)l of culture medium, and cells were further cultured for 1, 2, or 3 days.

**Lentivirus—**cDNA encoding HA-Glut4 (from Dr. Samuel Cushman, National Institutes of Health, Bethesda, MD) (8) was amplified by PCR and inserted at the AgeI site in frame upstream of the GFP reporter of the pRRL-PGK-GFP vector (from Dr. Rob C. Hoen, Leiden University Medical Center, Leiden, The Netherlands) (17). Lentivirus was prepared using the ViraPower lentiviral packaging mix and 293FT packaging cells following the manufacturer’s recommended protocol (Invitrogen). 3T3-L1 fibroblasts were infected with recombinant lentivirus essentially following recommended protocols (Invitrogen). Cells were plated at low density in 35-mm culture dishes and allowed to recover overnight in 2 ml of DMEM, 10% CS. The following day, the growth medium was replaced with infection medium (1 ml of HA-Glut4/GFP lentiviral supernatant diluted to 2 ml in DMEM, 10% CS, supplemented with 6 \( \mu \)g/ml Polybrene). Cells were incubated in infection medium for 24 h. Infection medium was then replaced with DMEM, 10% CS. Infected cells were allowed to reach 80% confluence and then cultured as described above. Infection efficiency was determined by flow cytometry. Under these conditions, ~80% of the cells expressed the HA-Glut4/GFP reporter construct.

**Flow Cytometry—**3T3-L1 cells (fibroblasts and differentiated adipocytes) were suspended by collagenase digestion (1 \( \mu \)g/\( \mu \)l in PBS plus 1% BSA, 20 \( \mu \)l/well, 5 min at 37°C or 30 min at 4°C) and gentle pipetting in a final volume of 200 \( \mu \)l of PBS plus 1% BSA. Suspended cells were filtered through a 100-\( \mu \)m cell strainer. Samples were analyzed by flow cytometry using a FACScan (BD Biosciences) equipped with argon (488 nm) and helium/neon (633 nm) lasers. Log intensities of scattered light (forward scatter (FSC) and side scatter (SSC); 488 nm excitation, helium/neon (633 nm) lasers) were collected. Data collection thresholds were set based on FSC and SSC profiles for undifferentiated fibroblasts (Fig. 1A) to omit small light scattering debris and machine noise from the analysis. Fluorescence detector gains
were set so that background autofluorescence in noninfected cells was collected in the first log decade of the detector range.

**Gating**—Differentiated 3T3-L1 cells contain three populations of light scattering objects: fully differentiated adipocytes, nondifferentiated fibroblasts, and necrotic cells that have died during the differentiation process (debris). The nondifferentiated fibroblasts were separated from the adipocytes and dead cell debris by setting an elliptical gate in SSC and FSC (low scatter cells (LSC); Fig. 1, A and D). Differentiated adipocytes were resolved from residual fibroblasts by setting an elliptical gate in SSC and FSC (high scatter cells (HSC); Fig. 1B). A second elliptical gate in SSC and FL3 autofluorescence was used to separate adipocytes from necrotic cell debris (Fig. 1C). Cells expressing the HA-Glut4/GFP reporter construct (LG4) were separated from cells that do not express the reporter (NV) using a gate in SSC and FL1 (Fig. 2). Gated populations (adipocytes, fibroblasts, and debris) were sorted using a Beckman Coulter EPICS Elite ESP sorter. Cells were collected on glass slides and imaged by differential interference contrast microscopy (Fig. 1E).

**Anti-HA Labeling in Fixed Cells**—Differentiated control (NV) and HA-Glut4/GFP lentivirus-infected (LG4) 3T3-L1 cells were suspended by collagenase digestion in PBS plus 2% BSA. The cell suspension was brought to 4% gluteraldehyde (methanol-free EM grade; Polysciences) by the addition of 16% gluteraldehyde, mixed by gentle pipetting, and incubated at room temperature for 5 min. Fix was quenched by the addition of PBS plus 5% BSA (5 times the volume of the gluteraldehyde-fixed cell suspension). Cells were washed three times by centrifugation (1000 x g for 3 min) and resuspended in PBS plus 2% BSA. Washed cells were permeabilized with IntraPrep (Beckman Coulter) according to the manufacturer’s instructions. Permeabilized cells were labeled with polyclonal anti-HA4 antibody (raised against the C-terminal 30 amino acids) for 1 h at room temperature, pelleted by centrifugation, and resuspended in PBS plus 2% BSA. Pelleting/resuspension was repeated three times to remove excess free anti-HA4 antibody. Cells were then labeled with Alexa647-conjugated anti-rabbit IgG secondary. Control cells were labeled with Alexa647 anti-rabbit IgG antibody alone. After labeling, cells were analyzed by flow cytometry (supplemental Fig. 1A).

**Purification and Labeling of Anti-HA**—Anti-HA 11 antibody (Covance) was purified from ascites on a fast flow protein A column (GE Health Sciences) following manufacturer’s recommended protocols. Briefly, ascites were diluted 10-fold in high salt loading buffer (3 M NaCl, 1.5 M glycine, pH 8.9) and injected onto the protein A column (pre-equilibrated in high salt loading buffer). The antibody-loaded protein A column was washed with 10 ml of high salt loading buffer. Bound anti-HA was eluted in 100 mM citric acid, pH 4.0. Eluted antibody was collected in 0.5-ml fractions into tubes containing 100 mM of 150 mM Tris, pH 8.1. Protein-containing fractions (absorbance 280 nm) were pooled and exchanged into PBS using a 5-ml desalting column (GE Health Sciences). Purified anti-HA was then labeled for 2 h at room temperature with constant stirring with succinimidyl ester Alexa647 (Invitrogen) following the manufacturer’s recommended protocols. Free dye was removed by exchange into PBS using a 5-ml desalting column (GE Health Sciences). Labeled antibody was stored in PBS at 4 °C. Absorbance spectroscopy was used to determine both the concentration of labeled antibody ([P]) and the concentration of Alexa647 ([Alexa]) in the eluate as follows.

\[
[P] = (\frac{A_{280} - (A_{647} \times 0.03)}{203,000 \text{ cm}^{-1} \text{ M}^{-1}}) \quad (\text{Eq. 1})
\]

\[
[\text{Alexa}] = \frac{A_{647}}{239,000 \text{ cm}^{-1} \text{ M}^{-1}} \quad (\text{Eq. 2})
\]

**Data Analysis**—The flow cytometric data were analyzed using FlowJo software (Tree Star Inc.). For each population of cells (adipocytes, fibroblasts, all cells) within each sample the geometric mean of FL4 was determined for both the HA-Glut4/GFP-positive and -negative (uninfected) cells. The mean fluorescence of the uninfected cells measures autofluorescence and nonspecific binding (BG). The background fluorescence varied with the time of incubation with anti-HA but was always less than 15% of total FL4 (supplemental Fig. 2D). The background-corrected mean fluorescence intensity (MFI) was calculated as MFI = FL4 − BG. The geometric mean of FL1 was determined for the HA-Glut4/GFP-positive cells. The FL1 MFI of uninfected cells (autofluorescence) was less than 5% of the FL1 MFI of infected cells (Fig. 2C); therefore, uncorrected FL1 MFIs were used in analysis. To correct for HA-Glut4/GFP expression levels, mean fluorescence ratios (MFRs) were calculated as MFR = MFI FL4/MFI FL1. This parameter is similar to calculations made by Martin et al. (3) and Govers et al. (4). To correct for differences in the Alexa647 labeling efficiencies in different batches of labeled anti-HA antibody, the MFRs were standardized to the calculated maximum MFR (MFR\text{max}) for cells treated with 100 nM insulin in each experiment. To calculate MFR\text{max}, the anti-HA uptake time course for insulin-stimulated cells was fit by a single exponential relaxation using Origin 7.5 software.
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RESULTS

3T3-L1 Adipocyte Cultures Are a Heterogeneous Population of Cells—Cultures of 3T3-L1 fibroblasts or differentiated adipocytes were analyzed by flow cytometry (Fig. 1). Dual parameter histograms of side scatter versus forward scatter and side scatter versus autofluorescence (FL3) were analyzed. Cultures of confluent fibroblasts contain a single population of cells with low scatter and low autofluorescence (Fig. 1A) (data not shown). In contrast, the cultures of differentiated adipocytes are mixed populations of cells (Fig. 1A, B and C). Two populations can be resolved based on scatter profiles: HSC and LSC. The high scatter objects can be further resolved into two populations, one with low autofluorescence and one with high autofluorescence (Fig. 1C). The high scatter, low autofluorescence cells are differentiated adipocytes. They contain lipid droplets (Fig. 1E) and express Glut4 (supplemental Fig. 1). The highly autofluorescent, high scatter objects are necrotic cell debris. They are very granular, with rough surfaces (Fig. 1E) and are highly autofluorescent in all detector channels (supplemental Fig. 1). The low scatter cells are indistinguishable in optical properties from confluent nondifferentiated 3T3-L1 cells (Fig. 1D). These are residual nondifferentiated fibroblasts. They contain no lipid droplets (Fig. 1E), express no Glut4 (supplemental Fig. 1), and spread and divide when replated on tissue culture dishes (data not shown). Propidium iodide staining showed that the adipocytes and fibroblasts are viable intact cells, whereas many of the necrotic cells are permeabilized (data not shown). Highly autofluorescent necrotic cells could also be detected by microscopy in intact monolayers of differentiated cells and were not solely the result of cell breakage during collagenase digestion and suspension of the cells (data not shown). The relative proportion of fibroblasts and necrotic debris to adipocytes was similar in samples within a single set of differentiated cells but varied from one batch of differentiated cells to the next. The number of residual fibroblasts tended to decrease with days post-initiation of differentiation, whereas the necrotic cells tended to increase. For analysis, elliptical gates in side scatter versus forward scatter were used to separate adipocytes from the residual fibroblasts. A second elliptical gate in side scatter versus FL3 was used to eliminate the highly autofluorescent necrotic cell debris from the adipocyte population.

3T3-L1 Adipocytes Are a Homogenous Population of Cells—An epitope-tagged HA-Glut4/GFP reporter construct was used to follow the traffic of Glut4 in differentiated adipocytes (8, 12). This construct is similar to that used by Martin et al. (3). The reporter was stably expressed in 3T3-L1 fibroblasts using recombinant lentivirus. Differentiated lentivirus-transduced cells were analyzed by flow cytometry (Fig. 2). Cells expressing the reporter are best resolved from uninfected cells in dual parameter histograms of GFP fluorescence (FL1) versus side scatter. Diagonal gates were used to resolve HA-Glut4/GFP expressing cells from noninfected cells as shown. Cells were infected with recombinant lentivirus at a titer sufficient to transduce ~80% of the cells. The uninfected cells serve as internal controls for nonspecific binding and autofluorescence in each sample. The FL1 fluorescence intensities of the infected cells (LG4) are 10–100 times greater than the mean fluorescence intensity of the noninfected cells (NV).

Overexpression of Glut4 has been reported to inhibit its basal intracellular retention (19, 20). To compare the level of expression of HA-Glut4/GFP with endogenous Glut4, infected and noninfected 3T3-L1 cells were differentiated, suspended by collagenase digestion, fixed, permeabilized, and labeled with an antibody that recognizes the carboxyl terminus of Glut4 (supplemental Fig. 1A). Infected and noninfected adipocytes (left) and fibroblasts (middle) were analyzed. Uninfected adipocytes express Glut4, whereas uninfected fibroblasts do not. Adipocytes expressing HA-Glut4/GFP have the same level of anti-Glut4 labeling as the uninfected adipocytes, indicating that the reporter is expressed at low levels relative to endogenous Glut4. Unlike the uninfected fibroblasts, the infected fibroblasts are positive for anti-Glut4 labeling, confirming that the anti-Glut4 antibody used in this analysis can recognize the epitope-tagged...
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Glut4/GFP fusion protein. There is a broad distribution of expression levels of endogenous Glut4 in adipocytes. There is also a broad distribution of HA-Glut4/GFP expression per cell. Both show approximately a log-normal distribution of fluorescence intensities (21). In infected adipocytes, there is no correlation between the anti-Glut4 labeling and GFP fluorescence, as would be expected if the HA-Glut4/GFP were expressed at low levels relative to endogenous Glut4. In contrast, there is a strong correlation between anti-Glut4 labeling and GFP fluorescence in infected fibroblasts, where the HA-Glut4/GFP accounts for all of the Glut4 expressed. These results are consistent with our previous experiments using recombinant adenovirus to express HA-Glut4/GFP in adipocytes (12).

To examine this in more detail, anti-HA binding was used to measure cell surface Glut4 content in adipocytes (Fig. 3). Differentiated lentivirus-transduced 3T3-L1 cells were incubated with or without insulin (100 nM, 30 min) and then labeled with Alexa647-conjugated anti-HA antibody at 4 °C and analyzed by flow cytometry. If overexpression of the HA-Glut4/GFP reporter were interfering with intracellular retention of Glut4, then basal cells expressing more HA-Glut4/GFP would retain less of the total transporter, and the surface-to-total ratio would increase as the expression level of the reporter increased. This was not observed. The basal surface-to-total ratio (calculated on a cell-by-cell basis) tends to decrease as the amount of HA-Glut4/GFP expressed increases (Fig. 3A). Therefore, even at the highest level of expression, the HA-Glut4/GFP does not saturate the basal retention machinery. This analysis also shows that although the differentiated cell culture is a mixed population of cells, the gated adipocytes behave as a single population. All adipocytes exhibit low surface-to-total ratios of Glut4 in basal cells and elevated surface-to-total ratios after insulin stimulation (mean FL4/FL1 increases 10–15-fold). In contrast, the transected fibroblasts exhibit a much broader distribution of basal surface HA-Glut4/GFP, with many cells showing elevated levels (supplemental Fig. 1B, middle).

Anti-HA Antibody Uptake in 3T3-L1 Adipocytes—To measure the kinetics of Glut4 traffic, differentiated 3T3-L1 cells expressing HA-Glut4/GFP were incubated at 37 °C in the continuous presence of Alexa647-conjugated anti-HA antibody for 10–240 min and analyzed by flow cytometry (3, 4, 9–11). The HA epitope is inserted into the first extracellular loop of the Glut4 reporter constructs. When anti-HA is added to the culture medium, cells expressing the reporter accumulate the antibody with time (Fig. 4). Although Glut4 resides in multiple compartments within the cell, whole cell anti-HA uptake can be fit by a single exponential relaxation of the following form,

\[
y(t) = Y_{\text{min}} + (Y_{\text{max}} - Y_{\text{min}})(1 - e^{-kt}) \tag{3}
\]

where \(Y(t)\) represents the total cell-associated anti-HA at time \(t\); \(Y_{\text{min}}\) is the amount of antibody bound at the earliest time point (10 min in our experiments); \(Y_{\text{max}}\) is the calculated maximum amount of antibody bound at \(t = \infty\); and \(k\) is the relaxation rate constant. If antibody binding is saturating, then \(Y_{\text{max}}\) is a measure of the size of the total cycling pool of Glut4. Martin et al. (3) and Govers et al. (4) both used the same monoclonal anti-HA antibody (HA.11; Covance) at a concentration of 50 µg/mL. To verify that this is a saturating concentration of antibody, uptake experiments were performed at antibody concentrations ranging from 1.25 to 80 µg/mL. MFIs of cell-associated Alexa647

3 In contrast to a normal (Gaussian) distribution, a log-normal distribution is not symmetrical about the mean when plotted on a linear scale. A greater proportion of cells express high levels of protein than low levels of protein. In addition, the variance is proportional to the level of expression (greater at higher expression levels). When log-normal data are plotted on a linear scale, the distribution contains a “tail” of high expressing cells (Fig. 2D). In contrast, when log-normal data are plotted on a logarithmic scale, expression levels are symmetrically distributed around the geometric mean (anti-log of the mean log fluorescence intensity) (Fig. 2C).

4 The fits are not improved by increasing the number of exponentials. Within the limit of resolution of these experiments, this indicates that either 1) Glut4 trafficking is a cycle and a single step is rate limiting for antibody uptake (this step might be different in basal versus insulin-stimulated cells) or 2) there are multiple steps that have similar rate constants (less than 2-fold difference) that therefore cannot be resolved. There also may be rapid steps that are largely complete before the first (10 min) time point.
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FIGURE 3. HA-Glut4/GFP is efficiently retained in basal cells. Differentiated, lentivirus-transduced 3T3-L1 (LG4) cells were serum-starved for 2 h, incubated with or without insulin (100 nM) for 30 min, and then labeled with Alexa647-conjugated anti-HA antibody at 4 °C. Side scatter, forward scatter, and FL1, FL3, and FL4 fluorescence (633 nm excitation/670 nm emission) intensities were collected for 20,000–30,000 cells. Gating was used to analyze only the adipocytes. A derived parameter, surface-to-total ratio (FL4/FL1), was calculated for each cell. A, basal cells; B, insulin-stimulated cells. All basal LG4 cells exhibit low surface HA-Glut4/GFP. Insulin stimulates robust translocation of HA-Glut4/GFP, increasing FL4/FL1 in all HA-Glut4/GFP-expressing cells. C, insulin increased the mean FL4/FL1 ratio 10–15-fold.

FIGURE 4. Kinetics of anti-HA uptake. Differentiated lentivirus-transduced 3T3-L1 (LG4) cells were serum-starved and incubated with insulin (100 nM, 30 min) and then further incubated with insulin and increasing concentrations of Alexa647-conjugated anti-HA antibody (1.25–80 µg/ml) for 10–240 min at 37 °C. Samples were analyzed by flow cytometry as described in Fig. 3. Data points are the average MFI ± S.E. from two separate experiments. A, anti-HA uptake. MFIs were corrected for autofluorescence and nonspecific uptake by subtracting MFI of uninfected cells in each sample. Lines, single exponential fits; MFI0 = MFImin + (MFImax − MFImin)(1 − e−kobs). B, transients from A were standardized to the calculated MFImax at each antibody concentration (MFI0 = MFImax(1 − e−kobs)). C, fractional saturation; calculated maximal amount of antibody bound at each antibody concentration (Bmax = MFImax − MFI0), standardized to the calculated maximal amount of antibody bound at [Ab] = ∞ (Bmax). D, concentration dependence of the kinetics of anti-HA uptake. Observed relaxation rate constants (kobs) were calculated from the single exponential fits in A. Below 40 µg/ml, kobs is approximately linearly dependent on [Ab] (dotted line); at 80 µg/ml, uptake is relatively insensitive to [Ab]. Filled symbol, kobs ± S.E. from eight additional uptake experiments done at 50 µg/ml anti-HA.

were calculated for the gated adipocytes in each sample. Plots of MFI versus time for each antibody concentration were fit by single exponential relaxations (Fig. 4A). The S.E. values for fit parameters were less than 10% of the predicted parameter values, the fit residuals were randomly distributed, and the R² for the fits were >0.98 in all cases (Fig. 4A and data not shown), indicating that the single exponential treatment is appropriate. The data could not be resolved into multiple relaxations by fitting with additional exponentials. MFImax and the observed relaxation rate constant (kobs) were calculated for each antibody concentration from these fits.

To calculate the EC50 of antibody binding, a plot of MFImax versus concentration of antibody (Fig. 4B) was fit by an equation for a hyperbola of the following form,

$$B(\text{Ab}) = B_{\text{max}}(\text{Ab})/(EC_{50} + [\text{Ab}])$$

(Eq. 4)

where $B(\text{Ab})$ is the calculated maximal amount of antibody bound at each antibody concentration ([Ab]; MFImax), and $B_{\text{max}}$ is the calculated maximum amount of antibody bound at [Ab] = ∞. Again, the S.E. values for the fit parameters were less than 10% of the predicted parameter values, the residuals were randomly distributed, and the R² value was >0.99, indicating an appropriate fit. The data could not be fit by multiple hyperbolic binding isotherms, indicating that a single antibody/antigen interaction controlled the amount of anti-HA associated with the cells. The EC50 for antibody binding was 3 µg/ml (20 nM). At 50 µg/ml, ~95% of the cycling HA-Glut4/GFP is bound by the antibody when steady state is reached.5

In these experiments, anti-HA accumulation is a function of both the rate of antibody binding and the rate of exchange of intracellular and cell surface Glut4. Both Martin et al. (3) and Govers et al. (4) make the explicit assumption that antibody binding is “instantaneous” and can therefore be ignored in their analysis. This treatment is only valid if accumulation is not dependent on

5 This assumes the concentration of antibody in the medium does not change during labeling, which was verified by sequentially labeling cells with the same labeling medium.
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does not decrease after cells were incubated for 120 min at 37 °C in medium without antibody. Thus, antibody accumulation is apparently irreversible on the time scale analyzed. As a final control, it was verified that antibody accumulation requires ATP synthesis in live cells and is inhibited by the metabolic poisons 2-deoxyglucose and azide (supplemental Fig. 2B).

Cell Surface Glut4 Levels Are Regulated by Static Retention in 3T3-L1 Adipocytes—Anti-HA uptake was measured in basal and insulin-stimulated cultures of 3T3-L1 cells (Fig. 5). MFIs of both cell-associated Alexa647 and GFP were calculated for each sample. To correct for cell-to-cell variation in HA-Glut4/GFP expression, MFIs were standardized to the MFRmax calculated from single exponential fits (MFR = MFI Alexa647/MFI GFP (FL4/FL1)). To compare separate experiments, data are standardized to the MFRmax calculated from single exponential fits (solid lines) of the 100 nM insulin data. This does not affect $k_{obs}$ (basal $k_{obs} = 0.0098 \pm 0.0038$ min$^{-1}$; insulin $k_{obs} = 0.039 \pm 0.0036$ min$^{-1}$).

The concentration of incubating antibody (the observed rate constant for antibody binding to antigen at the cell surface will increase linearly with [Ab]; $k_{obs} = k_{on}[\text{Ab}] + k_{off}$ based on the second order kinetics of antibody antigen binding). However, no data were presented to verify that this assumption can be made at 50 μg/ml anti-HA antibody. To demonstrate the effect of antibody concentration on the uptake kinetics, the amount of antibody associated with the cells was expressed as a fraction of the calculated maximal amount of antibody that will bind at steady state at each antibody concentration (% MFI max) and plotted versus time (Fig. 4C). This analysis clearly shows that the rate of accumulation depends on the concentration of antibody in solution. To look at this in more detail, a plot of $k_{obs}$ versus antibody concentration was analyzed (Fig. 4D). Between 1.25 and 40 μg/ml, $k_{obs}$ increases approximately linearly with respect to the concentration of incubating antibody. Thus, at 40 μg/ml antibody or less, accumulation is rate-limited by antibody binding. In contrast, uptake at 80 μg/ml is relatively insensitive to the concentration of incubating antibody. The rate of uptake at 50 μg/ml lies between the values at 40 and 80 μg/ml.

A second explicit assumption made by both Martin et al. (3) and Govers et al. (4) is that antibody binding is irreversible. To test this directly, the stability of cell-associated anti-HA in cells pulse-labeled with the antibody was measured (supplemental Fig. 2A). The amount of antibody associated with labeled cells in basal (squares) or insulin-stimulated (circles) 3T3-L1 adipocytes (differentiation day 10) as described in the legend to Fig. 4 using 50 μg/ml anti-HA. Data points are average MFR ± S.E. from three separate experiments. A, anti-HA uptake, MFR = MFI Alexa647/MFI GFP (FL4/FL1). To compare separate experiments, data are standardized to the MFRmax calculated from single exponential fits (solid lines) of the 100 nM insulin data. This does not affect $k_{obs}$ (basal $k_{obs} = 0.0098 \pm 0.0038$ min$^{-1}$; insulin $k_{obs} = 0.039 \pm 0.0036$ min$^{-1}$).

**FIGURE 5. Glut4 is regulated by static retention in 3T3-L1 adipocytes.** The kinetics of anti-HA uptake were measured in basal (squares) or insulin-stimulated (circles) 3T3-L1 adipocytes (differentiation day 10) as described in the legend to Fig. 4 using 50 μg/ml anti-HA. Data points are average MFR ± S.E. from three separate experiments. A, anti-HA uptake, MFR = MFI Alexa647/MFI GFP (FL4/FL1). To compare separate experiments, data are standardized to the MFRmax calculated from single exponential fits (solid lines) of the 100 nM insulin data. This does not affect $k_{obs}$ (basal $k_{obs} = 0.0098 \pm 0.0038$ min$^{-1}$; insulin $k_{obs} = 0.039 \pm 0.0036$ min$^{-1}$). B, comparison of free fits (solid line) with constrained fits where MFRmax was set to 0.9 (dotted line). C, predicted uptake (out to 30 h) based on the free and constrained fits of the basal uptake transient data. In the constrained fit, the $t_{1/2}$ for uptake is ~600 min (black dashed line). The $t_{1/2}$ value for basal uptake reported by Martin et al. is ~100 min (gray dashed line). D, cell-associated anti-HA after 600 min of uptake. Black dashed bar, amount expected based on the constrained fit; gray dashed bar, amount reported by Martin et al.

The internalization of Glut4 was analyzed out to 600 min (Fig. 5B). The amount of antibody associated with labeled cells for the fit parameters and decreased the S.E. values for each parameter were less than 10% of the predicted values, and the residuals were small and randomly distributed (data not shown). Therefore, as observed by Govers et al. (4), our analysis indicates that Glut4 is regulated by static retention.

In the dynamic equilibrium model, all of the Glut4 is actively cycling under both basal and insulin-stimulated conditions. Martin et al. (3) reported that the basal maximum binding was 90% of the insulin maximal binding. To verify that the static retention model most accurately describes our data, our free fit of the basal data was compared with a constrained fit in which basal MFRmax was set to 90% of the insulin MFRmax (Fig. 5B). Doing this dramatically increased the residuals and the S.E. values for the fit parameters and decreased the $R^2$ value of the fit. In addition, the most accurate fit of our data with a dynamic equilibrium model forces the observed rate constant of anti-HA accumulation to 0.001 ($t_{1/2}$ of ~600 min; Fig. 5C), significantly shorter than that observed by Martin et al. (3) ($k = 0.007, t_{1/2} = 100$ min. In our experiments, uptake was analyzed only out to 240 min, whereas in the work of Martin et al. (3), uptake was analyzed out to 600 min. However, even when analyzed after 10 h of uptake, the basal cells in our assay accumulated less than 30% of the anti-HA antibody that insulin-stimulated cells accumulated (Fig. 5D). In contrast, in the work of Martin et al. (3), the basal cells had accumulated 90% of the insulin-stimulated anti-HA antibody at this time.
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FIGURE 6. Insulin causes a dose-dependent increase in the size of the actively cycling pool of Glut4. Differentiated lentivirus-transduced 3T3-L1 (LG4) cells were serum-starved and stimulated with increasing concentrations of insulin for 30 min and then further incubated with insulin and 50 μg/ml Alexa647 anti-HA for 60 min at 4 °C (surface) (A) or 37 °C (cycling) (B). Samples were analyzed by flow cytometry as described in the legend to Fig. 3. Data points are average MFR ± S.D. from five (cycling) or 10 (surface) separate experiments. (C) Anti-HA uptake measured in basal cells (squares) or cells stimulated with 1 nM insulin (inverted triangles), 10 nM (triangles), or 100 nM (circles) insulin. Single exponential fits (solid lines) were used to calculate the total amount of cycling HA-Glut4/GFP (MFRmax) and kobs (E). Data points are average MFR ± S.E. from three separate experiments. No additional uptake was observed when fresh medium containing submaximal concentrations of insulin was added, indicating that the insulin is stable over the course of this experiment (data not shown).

Govers et al. (4) reported that insulin stimulates the quantal release of Glut4 from statically retained compartments. We also observe a dose-dependent increase in both cell surface Glut4 and the size of the actively cycling pool (Fig. 6, A and B). There is a 10–15-fold increase in cell surface labeling and a 5-fold increase in pool size with maximal insulin stimulation (100 nM) compared with basal cells. There is also a dose-dependent increase in the percentage of the total cycling pool that is found at the cell surface. In basal cells, 10–15% of the cycling pool is found at the plasma membrane (2–3% of the maximum insulin-stimulated pool), whereas in fully insulin-stimulated cells, 30–35% of the cycling pool is at the cell surface (data not shown). This shift reflects the 3–4-fold increase in the trafficking relaxation rate constant, kobs. To determine the dose dependence of kobs, the kinetics of antibody uptake were measured at 0, 1, 10, and 100 nM insulin (Fig. 6, C–E). Plots of MFR versus time were fit by single exponential relaxations. As reported by both Martin et al. (3) and Govers et al. (4), kobs at submaximal concentrations of insulin (1 and 10 nM) was intermediate between the values at 0 and 100 nM insulin (kobs = 0.024; Fig. 6E).

Replating Affects the Size of the Actively Cycling Pool—The differences in Glut4 trafficking observed in our experiments and those of Govers et al. (4) (static retention) and the experiments of Martin et al. (dynamic exchange) cannot be explained by differences in data analysis. They must therefore be due to differences in the experimental methodologies used. Differences in the HA-Glut4 reporters used (HA-Glut4/GFP versus HA-Glut4) as well as the method of reporter expression (transient expression by electroporation versus stable expression by retroviral transfection) have been investigated previously (3). Neither variable explained the differences in trafficking behaviors observed. However, several additional differences in the cell culture conditions in the two studies remained to be tested. In our experiments and those of Govers et al. (4), the anti-HA uptake experiments were done using confluent, quiescent monolayers of 3T3-L1 cells differentiated in a long protocol (3 days in differentiation medium, 3 days in insulin medium, cells used 2–5 days after removal of insulin). In the work of Martin et al. (3) the anti-HA uptake experiments were done using cells that were replated on glass coverslips after completing an abbreviated differentiation protocol (2 days in differentiation medium, 2 days in insulin medium). The cells were analyzed 1 or 2 days after replating. It is common practice to replate 3T3-L1 cells after differentiation for microscopy or electroporation experiments. However, the effect of this widely used technique on Glut4 traffic has not been reported.

To test the effect of replating on anti-HA uptake in 3T3-L1 adipocytes, differentiated cells were suspended by collagenase digestion, replated 1 day after the removal of insulin medium, and then cultured for an additional 2 days (Fig. 7). This treatment is similar to that used by Martin et al. (3). Parallel samples of control differentiated cells were maintained in confluent culture. Replating increased the size of the basal cycling pool of Glut4 to 80%, which is very similar to the 90% reported by Martin et al. In contrast, the relaxation rate constant for Glut4 cycling was not significantly affected (kobs = 0.014 min⁻¹ in replated cells versus 0.012 min⁻¹ in confluent adipocytes). These data indicate that the differences observed in trafficking
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Behavior by Martin et al. (3) and Govers et al. (4) are due at least in part to differences in cell culture conditions. Glut4 is Recycled to a Static Pool after Insulin Withdrawal—Under physiological conditions, insulin levels fluctuate as glucose levels fluctuate. In response to this fluctuation, Glut4 is rapidly inserted into the plasma membrane after insulin stimulation and is rapidly cleared from the plasma membrane when insulin is withdrawn. The Glut4 is recycled and is reused for subsequent stimulations with insulin (6). The increase in surface Glut4 to a new steady state level after insulin stimulation follows a single exponential relaxation, with a $t_{1/2}$ of 6.5 min (Fig. 8A). The surface Glut4 returns to basal levels after insulin withdrawal, with a $t_{1/2}$ of 20 min. Upon restimulation, the kinetics of transition from the basal to the insulin-stimulated state is very similar to the preinsulin kinetics ($t_{1/2} = 5$ min). To determine if Glut4 is recycled into the static pool, the size of the actively cycling pool of Glut4 was examined after insulin withdrawal (Fig. 8B). The total amount of Glut4 cycling through the plasma membrane in 1 h decreased rapidly upon removal of insulin. The kinetics of recovery were best fit by two concurrent exponential relaxations, one with a $t_{1/2} = 5$ min, and a second that relaxed much more slowly. To better characterize Glut4 trafficking after recovery, the full kinetics of Glut4 uptake were examined 3 h after insulin withdrawal (Fig. 8C). Glut4 is regulated by static retention 3 h after recovery from insulin stimulation. However, the total amount of cycling Glut4 in recovered cells remained elevated (0.48 versus 0.26 in the control cells). Interestingly, the $k_{obs}$ was also decreased by 2-fold in the recovered cells (0.0077 versus 0.015), so the total amount of Glut4 cycling through the plasma membrane in 1 h was similar in the control and recovered cells. In contrast, 12 h after removal of insulin, the kinetics of anti-HA uptake in recovered cells were identical to uptake in basal control cells (Fig. 8D).

**DISCUSSION**

We developed a flow cytometric assay to study Glut4 traffic in 3T3-L1 adipocytes. This was a modification of the anti-HA antibody uptake assays described by Govers et al. (3) and Martin et al. (4). In the flow assay, large numbers of cells are easily analyzed on a cell-by-cell basis. Thus, flow cytometry combines advantages of the whole plate assay described by Govers et al. (4) and the microscopy assay described by Martin et al. (3). Additional advantages of flow cytometry include sensitivity of detection, accurate noise correction, and ease of analyzing the large numbers of samples required for time course and dose response experiments. This method could easily be applied to other cycling surface antigens or receptors (13–15).

Using this assay, we found that cell surface Glut4 is regulated by static retention in confluent, quiescent 3T3-L1 adipocytes, in agreement with the results of Govers et al. (4). In contrast, Martin et al. (3) concluded that Glut4 was regulated solely by dynamic equilibrium. To understand the difference between our results and those of Martin et al. (3), we compared the kinetics of Glut4 trafficking in confluent differentiated cells and cells replated after differentiation. We found that Glut4 traffics differently under these two culture conditions. In confluent cultures, 80% of the Glut4 in basal 3T3-L1 adipocytes is retained in noncycling compartments (Fig. 5). This is most consistent with static retention, where GSVs behave like regulated secretory vesicles (4). In contrast, 80% of the Glut4 is cycling under basal conditions in replated cells (Fig. 7), consistent with dynamic exchange (3). We conclude that differences in the cell
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culture conditions can largely account for the differences in trafficking behavior observed by Martin et al. (3) and Govers et al. (4).

In addition to increasing the size of the actively cycling pool, insulin also increased the observed relaxation rate constant \((k_{obs})\) 3-fold, from 0.012 to 0.036 min\(^{-1}\). This is consistent with previous reports. Govers et al. (4) found that insulin increased the rate constant of Glut4 exocytosis from 0.014 to 0.03 min\(^{-1}\). Martin et al. (3) found that insulin increased \(k_{ex}\) from 0.007 to 0.05–0.07 min\(^{-1}\) (3). Using photoaffinity probes on primary adipocytes, Sato et al. (6) found that insulin increased \(k_{ex}\) from 0.012 to 0.032 min\(^{-1}\), whereas Jhun et al. (7) found that insulin increased \(k_{ex}\) from 0.024 to 0.078 min\(^{-1}\). Insulin caused a modest (2–7-fold) increase in \(k_{ex}\) in all studies.

The effect of insulin on endocytosis can also be estimated from the uptake data, if the partitioning coefficient, \(P\), can be accurately estimated \((P = [p]/[i] = k_{ex}/k_{en}\) where \([p]\) is the proportion of cycling Glut4 in the plasma membrane and \([i]\) is the proportion in intracellular compartments). At 50 \(\mu\)g/ml anti-HA, the amount of antibody associated with cells after 10 min of uptake (MFR\(_{10\text{min}}\)) is approximately equal to \([p]\) (within 10%; calculated using the equations in supplemental Fig. 3B). MFR\(_{max}\) gives the size of the total cycling pool, and \([i] = MFR_{max} - MFR_{10\text{min}}\). In our experiments, basal \(P = 0.12/0.88\), and insulin-stimulated \(P = 0.3/0.7\). Using these values and our calculated \(k_{ex}\), insulin has no effect on \(k_{en}\) (0.088 min\(^{-1}\) basal versus 0.084 min\(^{-1}\) insulin; \(k_{en} = k_{ex}/P\)). It is important to note that the partitioning coefficient must be calculated as a percentage of the total cycling pool of Glut4 under each condition, not the total amount of cellular Glut4. Inclusion of noncycling Glut4 (i.e. Glut4 in noncycling GSVs or in the biosynthetic or degradative pathways) will lead to an overestimate of \([i]\), an underestimate of \(P\), and an overestimate of \(k_{en}\). In our experiments, only 2.6% of the total cycling Glut4 (measured as MFR\(_{max}\) at saturating concentrations of insulin) is at the plasma membrane in basal cells. However, \([p] = 12\%\) in basal cells, because the basal cycling pool is only 22% of the total insulin-responsive cycling Glut4. After maximal insulin stimulation, 30% of the total cycling pool is at the plasma membrane. The net effect is that insulin increases cell surface Glut4 ~12-fold, through a 3-fold increase in \(k_{ex}\) and a 4-fold increase in \(k_{en}\).

In the work of both Martin et al. (3) and Govers et al. (4), the observed relaxation rate constants calculated from the single exponential fits were assumed to be \(k_{ex}\). This was based on the explicit assumption that binding at the cell surface was instantaneous and irreversible. If these assumptions are correct, then antibody labeling would be independent of antibody concentration [Ab], since the rate of binding increases linearly with increasing [Ab]. To test this, we measured uptake at [Ab], ranging from 1.25 to 80 \(\mu\)g/ml (Fig. 4). Although we found that 50 \(\mu\)g/ml is nearly saturating with respect to binding (it is 17 times the EC\(_{50}\) of binding), it is not saturating with respect to uptake. Under these conditions, the rate of antibody uptake will be a function of both binding and traffic, and \(k_{obs}\) is not simply \(k_{ex}\).

Both Martin et al. (3) and Govers et al. (4) interpret their data based on a “two-pool” model. In this model, the cycling Glut4 exchanges between the plasma membrane and intracellular compartments via exocytosis and endocytosis, with the rate constants \(k_{ex}\) and \(k_{en}\). Intracellular Glut4 is treated as a single kinetically homogeneous pool (in the work of Govers et al. (4), there is also a separate static pool). Others have used similar models (6, 7). To better understand the relationship between \(k_{obs}\) and \(k_{ex}\), we modified the model to include antibody binding (supplemental Fig. 3). This assay is a relaxation experiment. At \(t = 0\), no antibody is bound. When the system reaches equilibrium, antibody is bound to 95% of the HA epitopes both at the surface and inside of the cell at 50 \(\mu\)g/ml antibody. There are three reversible processes occurring: 1) intracellular Glut4 (i) is exchanging with plasma membrane Glut4 (p), with the rate constants \(k_{ex}\) and \(k_{en}\); 2) antibody is binding to and being released from HA-Glut4 at the plasma membrane, with the rate constants \(k_{on}\) and \(k_{off}\); 3) labeled HA-Glut4 at the plasma membrane (p*) is exchanging with the labeled intracellular pool (i*), with the rate constants \(k_{on}\) and \(k_{en}\) (supplemental Fig. 3A).

Antibody uptake in this model is described by a series of ordinary differential equations (supplemental Fig. 3B). Numerical methods were used to simulate uptake over a range of antibody concentrations, varying \(k_{ex}\), \(k_{en}\), and the partition coefficient, \(P = k_{ex}/P_{off} = k_{ex} \times K_{D}\). We fit a single exponential relaxation to the simulated data and assessed \(k_{obs}\) from this analysis. We found that \(k_{obs}\) was a function of \(k_{ex}\), \(k_{en}\), \(k_{on}\), and [Ab]. In contrast, varying \(k_{on}\) had little effect on \(k_{obs}\) but affected the maximal binding (through effects on \(K_{D}\)). When \(k_{ex}\) is >>\(k_{on}\), \(P\) is high, and most of the Glut4 is at the cell surface. Under these conditions, the rate of antibody accumulation is simply the rate of binding \((k_{obs} = k_{on}[Ab] + k_{off})\). When \(k_{ex}\) is >>\(k_{ex}\), \(P\) is low, and most of the Glut4 is intracellular. Under these conditions, \(k_{obs}\) is approximately \(k_{ex}\) at saturating concentrations of antibody. When \(P\) is set to the values calculated from our data (basal \(P = 0.12/0.88\); insulin \(P = 0.3/0.7\)), \(k_{obs}\) is a function of both binding and trafficking and does not simply equal \(k_{ex}\) (supplemental Fig. 3D). However, the \(k_{obs}\) versus [Ab] curve flattens out once \(k_{obs}\) is >>\(k_{ex}\) (supplemental Fig. 3F). At [Ab] close to this break point (40–80 \(\mu\)g/ml with this antibody), the rate of binding is close to the rate of exocytosis, and \(k_{obs}\) is a good approximation for \(k_{ex}\) (supplemental Fig. 3E).

What is \(k_{ex}\)? It is the apparent rate constant for delivery of HA-Glut4/GFP to a compartment where it can be labeled (at the plasma membrane, unoccupied). This is not necessarily the rate constant for fusion of GSVs to the plasma membrane, however. Although the cycling pool of Glut4 behaves as a kinetically homogenous population (uptake can be fit by a single exponential; two-pool model), this does not mean that there is only a single intracellular compartment through which Glut4 traffics. In an elegant modeling paper, Holman et al. (22) (also see Ref. 5) showed that a simple two-pool model in which endocytosis is essentially the reverse of exocytosis is insufficient to describe Glut4 trafficking. In the two-pool model, the rate constant for the transition from the basal to the insulin-stimulated state will be less than or equal to the rate constant for steady state uptake after insulin stimulation. It is not. The \(t_{1/2}\) for the basal to insulin-stimulated transition is 5–6 min (Fig. 8), whereas the \(t_{1/2}\) for uptake in insulin-stimulated cells is 17–19 min (Fig. 5). Holman et al. (22) showed that a “three-pool” model, in which Glut4 traffics through two sequential intracellular pools, the endosomes and a “sequestered” pool, could account for the discrep-
constants. In Holman’s three-pool model, Glut4 accumulates in GSVs in basal cells, because the rate constant for fusion of GSVs to the plasma membrane ($k_{\text{ex}}$) is much slower than the rate constant for transfer of Glut4 from endosomes to GSVs ($k_{\text{seq}}$). The basal-to-insulin transition is fast due to a burst of Glut4 release caused by a large (>100-fold) increase in $k_{\text{ex}}$. In contrast, steady state uptake is relatively slow, because it is a function of both $k_{\text{seq}}$ and $k_{\text{ex}}$.

Although the three-pool model describes the behavior of the static pool of Glut4, this model does not fully account for the observed kinetic data. It predicts a very slow $k_{\text{obs}}$ under basal conditions (0.001 min$^{-1}$), which increases >80-fold after insulin stimulation. However, in all of the kinetic studies reported, there is a pool of Glut4 in basal cells that is cycling much faster than this, with a $k_{\text{obs}} = 0.007$–0.024 min$^{-1}$. This $k_{\text{obs}}$ increases only 2–7-fold with insulin. The three-pool model also predicts a slow labeling of the Glut4 in the GSV pool in basal cells, which we do not observe; it does not predict the fast filling of a small pool of Glut4 (20–25%) that is observed (Figs. 5 and 9B). Finally, the three-pool model cannot account for the quantal release of Glut4 at submaximal insulin concentrations (Fig. 6). In contrast, static retention can account for the rapid transition kinetics with a $k_{\text{obs}}$ that increases only 3-fold (from 0.012 to 0.036 min$^{-1}$; Fig. 9C). It also accounts for the rapid labeling of a small pool in basal cells and the quantal release.

Static retention is a modification of the three-pool model (Fig. 9E). Rather than a sequential pathway where cycling Glut4 must traverse

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**FIGURE 9. Mathematical modeling.** Open symbols, simulated data; red filled symbols, experimental data. Lines, single exponential fits to simulated (solid) or experimental (dashed) data. A, numerical simulation of uptake based on the static retention model (see supplemental Fig. 3); B, numerical simulation of uptake based on the three-pool model (described by Holman et al. (22); the model was modified only to include antibody binding). C, numerical simulation of cell surface Glut4 during the basal to insulin-stimulated transition based on the static retention model; $[p]_o$ set to 2.6% (basal level), $[i]_o$ set to the total insulin-stimulated cycling pool $−[p]_o$ (100% − 2.6%), and $k_{\text{ex}}$ set to $6.2 \times 10^{-4}$ s$^{-1}$ (insulin $k_{\text{ex}}$). No binding occurs in this experiment. This simulates instantaneous release of the static pool of Glut4 into the cycling pool and an instantaneous increase in $k_{\text{ex}}$. D, numerical simulation of cell surface Glut4 levels during the insulin-stimulated to the basal transition after insulin withdrawal; $[p]_o$ set to 30% (insulin level), $[i]_o$ set to the total insulin-stimulated cycling pool $−[p]_o$ (100% − 30%), $k_{\text{ex}}$ set to $1 \times 10^{-4}$ s$^{-1}$ (1/5 basal $k_{\text{ex}}$), and $k_{\text{seq}}$ set to $14.7 \times 10^{-4}$ s$^{-1}$ (basal $k_{\text{seq}}$). E, comparison of the three-pool model versus static retention. In both models, Glut4 traverses three pools: the PM, endosomes (end), and GSVs. Glut4 is trafficked to the PM with a rate constant $k_{\text{ens}}$ Glut4 is retrieved from the PM with the rate constant $k_{\text{ex}}$, and Glut4 is recycled from the endosomes to GSVs by sequestration ($k_{\text{seq}}$). However, in the three-pool model, $k_{\text{ens}}$ is the rate constant for the fusion of GSVs to the PM, Glut4 traffic to the cell surface only through GSVs, and $k_{\text{seq}}$ is fast and does not change. In static retention, Glut4 traffic to the PM through the endosomal cycling pool with the rate constant $k_{\text{ens}}$, insulin transiently stimulates quantal release of Glut4 from GSVs into the cycling pool, and sequestration is transiently elevated in response to insulin withdrawal ($k_{\text{seq}}$) is very slow in both basal and insulin-stimulated cells).
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quantal release (the higher the concentration of insulin, the more that is released). Interestingly, quantal release indicates that insulin only transiently activates the release mechanism; the same Glut4 cycles repeatedly through the system, and no additional Glut4 is released with prolonged insulin stimulation. Importantly, the data indicate that the cycling and static pools do not mix in either basal or insulin-stimulated cells. This indicates that under both conditions, the repackaging of Glut4 from the cycling endosomal system into static GSVs ($k_{ex}$) is very slow or blocked. However, Glut4 clearly recycles to the static GSVs after insulin withdrawal (Fig. 8). This indicates that insulin withdrawal transiently activates the sequestration mechanism to allow released Glut4 to return to the static pool.

As a final test of this assay, the kinetics of Glut4 cycling after insulin withdrawal were analyzed (Fig. 8). The amount of Glut4 at the plasma membrane decreased rapidly, following a single exponential relaxation with a $t_{1/2} = 20$ min (Fig. 8A). The total Glut4 cycling through the plasma membrane in 1 h also decreased very rapidly but was more accurately described by two concurrent exponential relaxations, one with a $t_{1/2} = 5$ min, and a second that relaxed slowly (Fig. 8B). At 3 h after insulin withdrawal, a significant proportion of the Glut4 has been repackaged into the static pool (Fig. 8C). However, the cycling pool is still 2-fold larger in the recovering cells than in control basal cells ($MFR_{max} = 0.48$ versus 0.26). Interestingly, the $k_{ex}$ in the recovering cells is decreased by ~2-fold relative to control basal cells ($0.0077$ versus $0.015$ min$^{-1}$). 12 h after insulin withdrawal, the static pool is fully recovered. These observations suggest that when insulin levels decrease, the transporter is rapidly cleared from the cell surface via rapid adjustments in $k_{ex}$ and $k_{en}$. Although the static pool slowly reforms, Glut4 is retained within cycling endosomes by rapid endocytosis and slow exocytosis. In support of this idea, a rapid change in $k_{ex}$ from the maximal insulin-stimulated rate to one-half the basal rate can fully account for the observed rapid rate of clearance of Glut4 from the plasma membrane after insulin withdrawal, despite slow repackaging into GSVs (Fig. 9D). Once the static pool fully reforms, however, static retention maintains Glut4 in a noncycling compartment, and the relative rates of exocytosis and endocytosis have less effect on Glut4 distribution. In this mechanism, dynamic exchange would regulate Glut4 retention when serum insulin concentrations fluctuate rapidly, whereas static retention would sequester Glut4 when insulin concentrations fluctuate more slowly. Thus, both mechanisms may play a role under physiological conditions.

The relative contributions of static retention and dynamic exchange on Glut4 trafficking in primary adipocytes and in intact tissue remain to be determined. One of the most complete descriptions of Glut4 trafficking in intact adipose tissue is an immunoelectron microscopy study of Glut4 localization in brown adipose tissue isolated from basal or insulin-treated rats by Slot et al. (23). Under basal conditions, less than 1% of the membrane-associated Glut4 labeling was observed at the plasma membrane. More than 80% of the Glut4 was found in small vesicles and tubules and the trans-Golgi network, where it did not co-localize with endocytic markers. After insulin stimulation, 35–40% of the Glut4 was at the plasma membrane, and the Glut4 was enriched in endosomes co-localized with endocytic markers. It remains to be determined whether the nonendocytic compartments containing Glut4 are static or cycling compartments and whether this changes in response to insulin. However, if dynamic equilibrium were the sole mechanism determining the distribution of Glut4 in these cells, then $k_{ex}$ would have to be 99-fold slower than $k_{en}$ in basal cells ($P = 0.009/0.99$), and $k_{ex}/k_{en}$ would have to be 66-fold lower in basal cells than insulin-stimulated cells ($P = 0.35/0.65$). The published kinetic data indicate that there is a pool of Glut4 that is cycling much faster than this in basal primary cells. In contrast, if it is assumed that cycling Glut4 is found in the plasma membrane, clathrin-coated pits/vesicles, and early endosomes, then 3.9% is cycling under basal conditions, and 48.9% is cycling after maximal insulin stimulation (there is a 12.5-fold increase in the cycling pool). More consistent with the observed kinetic data, $k_{ex}/k_{en}$ would be only 3.1-fold lower in basal cells than in insulin-stimulated cells ($P = 0.009/0.39$ basal versus 0.35/0.489 after insulin). This yields a 38.75-fold increase in cell surface Glut4 (12.5 $\times$ 3.1), in excellent agreement with the observed 39-fold increase (from 0.9 to 35%). The true trafficking behavior of Glut4 is likely to lie between these two models.

Our analysis indicates that Glut4 trafficking is regulated at multiple levels that differ in importance depending on culture conditions. An important finding from this work is that cell-to-cell or cell-to-matrix contacts affect Glut4 trafficking. It will be important to assess the impacts of tissue organization on other aspects of glucose and lipid metabolism as well.

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REFERENCES

1. Bryant, N. J., Gover, R., and James, D. E. (2002) Nat. Rev. Mol. Cell Biol. 3, 267–277
2. Foster, L. J., and Klip, A. (2000) Am. J. Physiol. 279, C877–C890
3. Martin, O. J., Black, D. A., and McGraw, T. E. (2006) J. Biol. Chem. 281, 484–490
4. Gover, R., Coster, A. C., and James, D. E. (2004) Mol. Cell Biol. 24, 6456–6466
5. Coster, A. C., Gover, R., and James, D. E. (2004) Traffic 5, 763–771
6. Sato, S., Nishimura, H., Clark, A. E., Kozka, I. J., Vannucci, S. J., Simpson, I. A., Quon, M. J., Cushman, S. W., and Holman, G. D. (1993) J. Biol. Chem. 268, 17820–17829
7. Jhan, B. H., Rampal, A. L., Liu, H., Lachaal, M., and Jung, C. Y. (1992) J. Biol. Chem. 267, 17710–17715
8. Dawson, K., Aviles-Hernandez, A., Cushman, S. W., and Malide, D. (2001) Biochem. Biophys. Res. Commun. 287, 445–454
9. Karylowksi, O., Zeigerer, A., Cohen, A., and McGraw, T. E. (2004) Mol. Biol. Cell 15, 870–882
10. Zeigerer, A., Lampson, M. A., Karylowksi, O., Sabatini, D. D., Adesnik, M., Ren, M., and McGraw, T. E. (2002) Mol. Biol. Cell 13, 2421–2435
11. Zeigerer, A., McBryar, M. K., and McGraw, T. E. (2004) Mol. Biol. Cell 15, 4406–4415
12. Muretta, J. M., Romaneskaia, I., Cassiday, P. A., and Mastick, C. C. (2007) J. Cell Sci. 120, 1168–1177
13. Cain, C. C., and Murphy, R. F. (1988) J. Cell Biol. 106, 269–277
14. Cain, C. C., Sipe, D. M., and Murphy, R. F. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 544–548
15. Cain, C. C., Wilson, R. B., and Murphy, R. F. (1991) J. Biol. Chem. 266, 11746–11752
16. Frost, S. C., and Lane, M. D. (1985) J. Biol. Chem. 260, 2646–2652
17. Carlotti, F., Bazuine, M., Kekarainen, T., Seppen, J., Pognonec, P., Maasen, J. A., and Hoeben, R. C. (2004) Mol. Ther. 9, 209–217
18. Gibbs, E. M., Allard, W. J., and Lienhard, G. E. (1986) J. Biol. Chem. 261, 16597–16603
19. Carvalho, E., Schellhorn, S. E., Zabolotny, J. M., Martin, S., Tozzo, E., Peroni, O. D., Houseknecht, K. L., Mundt, A., James, D. E., and Kahn, B. B. (2004) J. Biol. Chem. 279, 21598–21605
20. Waters, S. B., D’Auria, M., Martin, S. S., Nguyen, C., Kozma, L. M., and Luskey, K. L. (1997) J. Biol. Chem. 272, 23323–23327
21. Furusawa, C., Suzuki, T., Kashiwagi, A., Yomo, T., and Kaneko, K. (2005) Biophysics (Japan) 1, 25–31
22. Holman, G. D., Lo Leggio, L., and Cushman, S. W. (1994) J. Biol. Chem. 269, 17516–17524
23. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123–135