GLUT4 Distribution between the Plasma Membrane and the Intracellular Compartments Is Maintained by an Insulin-modulated Bipartite Dynamic Mechanism

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The GLUT4 glucose transporter is predominantly retained inside basal fat and muscle cells, and it is rapidly recruited to the plasma membrane with insulin stimulation. There is controversy regarding the mechanism of basal GLUT4 retention. One model is that GLUT4 retention is dynamic, based on slow exocytosis and rapid internalization of the entire pool of GLUT4 (Karylowski, O., Zeigerer, A., Cohen, A., and McGraw, T. E. (2004) Mol. Biol. Cell 15, 870–882). In this model, insulin increases GLUT4 in the plasma membrane by modulating GLUT4 exocytosis and endocytosis. The second model is that GLUT4 retention is static, with ~90% of GLUT4 stored in compartments that are not in equilibrium with the cell surface in basal conditions (Govers, R., Coster, A. C., and James, D. E. (2004) Mol. Cell Biol. 24, 6456–6466). In this model, insulin increases GLUT4 in the plasma membrane by releasing it from the static storage compartment. Here we show that under all experimental conditions examined, basal GLUT4 retention is by a bipartite dynamic mechanism involving slow exocytosis and rapid internalization. To establish that the dynamic model developed in studies of the extreme conditions of >100 nM insulin and no insulin also describes GLUT4 behavior at more physiological insulin concentrations, we characterized GLUT4 trafficking in 0.5 nM insulin. This submaximal insulin concentration promotes an intermediate effect on both GLUT4 exocytosis and endocytosis, resulting in an intermediate degree of redistribution to the plasma membrane. These data establish that changes in the steady-state surface/total distributions of GLUT4 are the result of graded, insulin-induced changes in GLUT4 exocytosis and endocytosis rates.

Insulin regulates glucose uptake into fat and muscle cells by eliciting a redistribution of the GLUT4 from its predominantly intracellular localization to the plasma membrane (1–4). This redistribution corresponds to a shift from less than 5% GLUT4 in the plasma membrane in basal conditions to about 50% of GLUT4 in the plasma membrane in the presence of insulin. The mechanism by which insulin causes the redistribution of GLUT4 has not been elucidated.

Intracellular retention of GLUT4 in basal conditions, by establishing a low basal glucose flux into muscle and fat, has a role in maintaining basal glucose homeostasis and in maximizing the potential range of stimulatory effects of insulin on glucose uptake. Consequently, understanding the mechanism of basal retention is critical for understanding regulated glucose transport into muscle and adipose.

We have measured the kinetics of GLUT4 trafficking using a GLUT4 construct with an HA epitope in the first exofacial loop and GFP fused to the carboxyl terminus (HA-GLUT4-GFP) expressed in 3T3-L1 adipocytes by electroporation (5–8). We found that retention of HA-GLUT4-GFP is maintained by a dynamic, bipartite mechanism involving slow exocytosis and rapid internalization (8). In this mode of retention, intracellular GLUT4 is in equilibrium with the cell surface in basal conditions. The results of a number of other studies support this dynamic, bipartite retention model in adipocytes (9–12) and muscle cells (13, 14).

An alternative retention model has recently been proposed in which intracellular retention of GLUT4 is maintained by a “static” mechanism, with the vast majority of GLUT4 not in equilibrium with the plasma membrane in basal conditions (15).

These different models suggest different molecular mechanisms for the basal retention of GLUT4. In the dynamic model, insulin regulates steady-state GLUT4 distribution by affecting the kinetics of its cycling (both exocytosis and endocytosis). In contrast, the static retention model suggests that insulin regulates the pool size of GLUT4 that moves to the plasma membrane and consequently that basal exclusion of GLUT4 from the plasma membrane is achieved because the majority of GLUT4 is fixed inside the cell.

To understand insulin regulation of GLUT4 trafficking, it is imperative that we understand the retention mechanism. In addition, this controversy raises questions about the methods used to analyze GLUT4 trafficking, warranting a detailed comparison of these widely used methods.

Here we compare the different methods used to examine GLUT4 trafficking. We find that GLUT4 continually cycles between intracellular compartments and the plasma membrane in the basal conditions, regardless of transfection method (electroporation versus retroviral infection) and the GLUT4 reporter used (HA-GLUT4 versus HA-GLUT4-GFP). These data are in agreement with our previous results, and they provide additional support for the dynamic, bipartite retention model (8).

In the second part of this paper, we explore the mechanism of insulin regulation of GLUT4 trafficking from a different perspective. In order to provide a view of the transition between the two end points of basal (no insulin) and maximal stimulation (200 nM insulin), we examined the
 trafficking kinetics of GLUT4 at submaximal insulin concentrations. Here we report that in submaximal insulin (0.5 nM), the changes in the rates of GLUT4 exocytosis and internalization are intermediate between basal and maximal insulin. Thus, the dynamic, bipartite GLUT4 trafficking model describes the behavior of GLUT4 at 0.5 nM insulin, a more physiological insulin concentration (16, 17).

**EXPERIMENTAL PROCEDURES**

*Ligands and Chemicals—*Fluorescent secondary antibodies were purchased from Jackson ImmunoRabs, Inc. (West Grove, PA). All chemicals were purchased from Sigma. Mouse anti-HA monoclonal antibody (HA.11) was purified from ascites (Covance, Berkeley, CA) using a protein G affinity column (Amersham Biosciences). The concentration of HA.11 required to saturate the HA epitope of HA-GLUT4-GFP was determined for each preparation of antibody by measuring cell-associated HA.11 antibody after a 10-min pulse at 37 °C. Typically, 50 μg/ml HA.11 was a saturating concentration. HA.11 and transferrin were purchased from Jackson Immunolabs, Inc. (West Grove, PA). All chemicals were purchased from Sigma. Mouse anti-HA monoclonal antibody (HA.11) was a saturating concentration. HA.11 and transferrin were purchased from Jackson Immunolabs, Inc. (West Grove, PA). Mouse anti-HA monoclonal antibody (HA.11) was purified from ascites (Covance, Berkeley, CA) using a protein G affinity column (Amersham Biosciences). The concentration of HA.11 required to saturate the HA epitope of HA-GLUT4-GFP was determined for each preparation of antibody by measuring cell-associated HA.11 antibody after a 10-min pulse at 37 °C. Typically, 50 μg/ml HA.11 was a saturating concentration. HA.11 and transferrin were labeled with Cy3 and Cy5, respectively, according to the manufacturer’s instructions (Biological Detection System, Pittsburg, PA). Monoclonal anti-GlUT4 antibody was a gift from Sam Cushman.

**Cell Culture—**3T3-L1 cells were differentiated into adipocytes as described previously (18). 3T3-L1 adipocytes were transfected by electroporation 5 days after differentiation and plated into coverslip bottom dishes (6). Stable expression of HA-GLUT4 was achieved using the retroviral expression vector pBABE-Puro (19). The pBABE HA-GLUT4 construct was a gift from David James. Retroviral stocks of HA-GLUT4 were generated using AmphiPack-293 cells (Clonetech, Mountain View, CA). 3T3-L1 fibroblasts were infected with the virus in the presence of 4 μg/ml Polybrene. After a 24-h recovery period, the cells were selected with 2 μg/ml puromycin for 1 week. The selected cells were grown to confluence and differentiated. On day 5 after differentiation, cells were transferred to coverslip bottom dishes. The cells were maintained at 37 °C in 5% CO2/air during growth and differentiation and for all incubations in the kinetic experiments. All experiments were conducted on cells 6 days after differentiation unless noted otherwise.

**Fluorescence Quantification—**All images were collected on a DMIRB inverted microscope (Leica Microsystems, Deerfield, IL) with a cooled charge-coupled device 12-bit camera (Princeton Instruments, West Chester, PA). Images for quantification of retrovirally infected cells were taken with a 10 × 0.30 numerical aperture dry objective. All other images were taken using a 40 × 1.25 numerical aperture oil immersion objective. Exposure times for each fluorescence channel were chosen such that >95% of the image pixel intensities were below camera saturation. Exposure times were kept constant within each experiment. Image quantification was done as described previously using Metaphor (Universal Imaging, West Chester, PA) image processing software (20, 21). All images were background-corrected using nonexpressing cells. For transiently transfected cells, the background cells were chosen from within the same dish as the expressing cells. For stably transfected cells expressing HA-GLUT4, the background cells were collected from separate dishes of untransfected cells that were treated with antibodies in the same way as the transfected cells.

**Indirect Immunofluorescence—**For indirect immunofluorescence, cells were fixed with 3.7% formaldehyde, incubated with primary antibody in 150 mM NaCl, 20 mM HEPES, 1 mM CaCl2, 5 mM KCl, and 1 mM MgCl2, pH 7.2 (medium 1) with 250 μM saponin (for permeabilization) and 1% calf serum for 30 min at 37 °C, washed and incubated with fluorescent secondary antibody in medium 1/saponin/calf serum.

**Measurement of Total HA-GLUT4 Expression in Transiently Transfected Cells—**Adipocytes transfected by electroporation with HA-GLUT4 were incubated in serum-free Dulbecco’s modified Eagle’s medium with 220 mM bicarbonate and 20 mM HEPES, pH 7.4 (DMEMBB), supplemented with Cy3-HA.11 antibody and 170 nM insulin for 2 h to label the entire cycling pool of GLUT4. The cells were washed to remove unbound antibody and insulin and returned to basal during a 2-h incubation in DMEMBB (8). The cell-associated Cy3 fluorescence is a measure of the total HA-GLUT4 expressed per cell (i.e. HA-GLUT4 recruited to the plasma membrane during the 2-h preincubation with Cy3-HA.11 and insulin). HA.11 binding does not alter GLUT4 trafficking, and it remains bound to the epitope during transit through intracellular compartments (5–8).

**GLUT4 Surface/Total Distribution—**The procedure for measurement of HA-GLUT4-GFP translocation to the plasma membrane has been described (5, 6). For translocation measurements of HA-GLUT4, the total HA-GLUT4 expressed by electroporation in individual adipocytes was measured by uptake of Cy3-HA.11 as described above. Cells were then either stimulated for 15 min with 170 nM insulin and fixed or immediately fixed in 3.7% formaldehyde (basal). HA-GLUT4 on the surface was measured by incubating the fixed cells with a Cy5-labeled goat anti-mouse antibody. The Cy5 fluorescence associated with surface-exposed HA.11 was normalized to total exogenously expressed GLUT4 per cell using the Cy3-HA.11. The surface/total ratio of exogenous GLUT4 was determined for 20 cells/condition/experiment.

**GLUT4 Trafficking Kinetics in Submaximal Insulin—**We found that a large volume of insulin solution to cell density was required (e.g. 2 ml/~5 × 106 cells) to maintain the steady-state surface/total distribution of GLUT4 in 0.5 nM insulin (supplemental Fig. 1A). The response of cells to insulin, as measured by redistribution of GLUT4 to the plasma membrane, declined over time when cells were incubate in small volumes of 0.5 nM insulin (100 μl/~5 × 106 cells; supplemental Fig. 1A). This effect was not observed when cells were incubated in small volumes of 200 nM insulin. The addition of a protease inhibitor mixture (catalog no. P1860; Sigma) (containing aprotinin, bestatin, E-64, leupeptin, and pepstatin) to the solution abrogated the decay of the 0.5 nM response (supplemental Fig. 1B). The protease inhibitor mixture had no effect on the response to 200 nM insulin (supplemental Fig. 1C). The protease inhibitor mixture was used at a 1:200 dilution. It is likely that a protease produced by the cells is degrading the insulin. The decline over time of the response of cells to insulin does not occur when incubating either with large volumes of insulin solution or with small volumes of high insulin concentration. In the experiments measuring the trafficking of GLUT4 in submaximal insulin, the protease inhibitor was added to the DMEMBB medium containing HA.11 antibody for incubations in a small volume longer than 45 min.

**GLUT4 Exocytosis—**For experiments comparing the efflux of HA-GLUT4 and HA-GLUT4-GFP expressed by electroporation, the entire pool of exogenous GLUT4 was prelabeled with Cy3-HA.11 as described above. Cells were incubated in DMEMBB and Cy5-goat anti-mouse antibody (basal) or prestimulated with 170 nM insulin for 15 min and then incubated in DMEMBB and Cy5-goat anti-mouse antibody (insulin). For each cell, the cell-associated Cy5-goat anti-mouse antibody fluorescence was normalized to the Cy3-HA.11 fluorescence. The Cy5/Cy3 ratio was determined for at least 20 cells/time point/experiment.

For measurement of exocytosis of HA-GLUT4 expressed by retroviral infection, adipocytes were preincubated in DMEMBB for 2 h and then incubated in DMEMBB and HA.11 antibody in the presence or absence of 170 nM insulin. The amount of HA.11 taken up in a given amount of time was determined in fixed, saponin-permeabilized cells by...
staining with Cy3-goat anti-mouse antibody. Cell-associated HA.11 was determined for 20 fields of confluent cells (about 200 cells)/time point/condition. No correction for expression level was made because the average expression level/field was fairly constant.

For experiments comparing the GLUT4 efflux in 0.5 nm versus 200 nm insulin, 3T3-L1 adipocytes electroporated with HA-GLUT4-GFP were incubated in DMEMBB for 2 h. Cells were prestimulated with 2 ml of insulin (200 or 0.5 nm) for 1 h. Following this, cells were incubated with a saturating concentration of HA.11 antibody in the presence of protease inhibitor mixture and insulin. The amount of HA.11 taken up in a given amount of time was determined by permeabilizing (250 μM saponin) the cells and staining with Cy3-goat anti-mouse antibody. Cy3 fluorescence was normalized for expression level of GLUT4 using GFP.

Endocytosis—Internalization was measured using a modification of a previously published method (7). Briefly, cells were incubated in DMEMBB for 2 h, following which they were incubated in 2 ml of medium containing 0, 0.5, or 200 nm insulin for 1 h. The cells were then incubated in medium containing the 0.5 or 200 nm insulin and a saturating concentration of HA.11 for 3, 5, 7, or 9 min or in medium with no insulin and a saturating concentration of HA.11 for 10, 15, 20, or 30 min. After each time point, cells were fixed with 3.7% formaldehyde, and surface-exposed HA.11 was stained with a saturating concentration of Cy3-goat anti-mouse antibody. The cells were then permeabilized with 250 μM saponin, and stained with Cy5-goat anti-mouse antibody to reveal all internal HA.11 (the Cy5-goat anti-mouse antibody used binds the same epitopes as the Cy3 antibody and therefore does not bind to the surface-exposed epitopes already bound by Cy3-goat anti-mouse antibody).

For transferrin receptor (TR) internalization, cells electroporated with human transferrin receptor were incubated in DMEMBB for 210 min, with 200 nm insulin included for the last 30 min of incubation for half of the samples. Cells were incubated in DMEMBB and 10 μg/ml Cy3-transferrin with or without 200 nm insulin for 3, 5, 7, or 9 min. Cy3-transferrin bound to TR on the cell surface was removed by alternating acidic and neutral washes on ice (8). Cy5-transferrin remaining cell-associated after these washes was the portion internalized during the incubations. The cells were then fixed with 3.7% formaldehyde, and TR on the plasma membrane was stained with monoclonal anti-TR antibody (B3/25). Cy3-goat anti-mouse antibody was used to detect the B3/25. For both GLUT4 and TR internalization experiments, the slope of the line generated by plotting the time-dependent increase in the ratio of the internal to surface fluorescence is proportional to the internalization rate.

RESULTS

The Mechanism of Basal GLUT4 Retention: Dynamic or Static Retention?—It has previously been shown that the targeting of GLUT4 to the insulin-regulated pathway can be saturated at high levels of GLUT4 expression (22, 23). To determine whether targeting of HA-GLUT4-GFP to the specialized pathway is saturated at the expression levels achieved by electroporation, we compared HA-GLUT4-GFP expression with endogenous GLUT4 expression. The amount of total GLUT4/cell in 3T3-L1 adipocytes transiently transfected with HA-GLUT4-GFP was determined by quantitative indirect immunofluorescence (IF) using an antibody against the carboxyl cytoplasmic domain of GLUT4. The transfected cells expressing HA-GLUT4-GFP were identified by GFP fluorescence, and in these cells, total GLUT4 detected by IF is the sum of endogenous GLUT4 and HA-GLUT4-GFP. In cells that did not express HA-GLUT4-GFP (no GFP fluorescence), the IF signal reflects the amount of endogenous GLUT4 alone. The overall morphology of the GLUT4 compartments was perinuclear with small punctate structures around the periphery of the cell and was unaltered by the expression of exogenous GLUT4 (Fig. 1A). Total GLUT4 in cells expressing HA-GLUT4-GFP was about 2-fold greater than in cells expressing endogenous GLUT4 alone (Fig. 1B). These data indicate that, on average, HA-GLUT4-GFP was expressed at the same level as endogenous GLUT4. Analysis of the data on a cell-by-cell basis revealed that: (a) the range of expression of total GLUT4 in cells expressing HA-GLUT4-GFP was similar to that observed for endogenous GLUT4; (b) in both cases, the distribution of GLUT4 expression was normal; and (c) only a small fraction of the transfected cells (less
than 10%) expressed total GLUT4 to a level that was greater than the range of endogenous expression in untransfected cells (Fig. 1C).

For the IF to be a valid measure of the relative expression of HA-GLUT4-GFP and endogenous GLUT4, the affinity of the antibody for the two proteins must be similar. We examined the effect of varying the antibody concentration on the ratio of HA-GLUT4-GFP and endogenous GLUT4 to endogenous GLUT4. If the affinity of the anti-GLUT4 antibody is lower for HA-GLUT4-GFP than for endogenous GLUT4, this ratio will decrease as the concentration of antibody decreases. Decreasing the antibody concentration did not significantly decrease the ratio (Fig. 1D), indicating that the affinity of the anti-GLUT4 antibody used is similar for HA-GLUT4-GFP and for endogenous GLUT4.

To directly examine the relationship between the amount of HA-GLUT4-GFP expressed and its intracellular retention, we correlated, on an individual cell basis, the basal surface-to-total ratio (Cy3/GFP) of HA-GLUT4-GFP to the amount of HA-GLUT4-GFP expressed (Fig. 1E). There was no increase in the Cy3/GFP ratio in the cells that express more of the HA-GLUT4-GFP (higher GFP signal), as would be expected if, at levels of expression, retention was saturated. These data demonstrate that HA-GLUT4-GFP expressed by electroporation does not saturate the basal retention mechanism.

HA-GLUT4-GFP is a convenient reporter for endogenous GLUT4 because the GFP provides a way to normalize the distribution of HA-GLUT4-GFP to the expression level per cell (6, 21). However, the carboxyl terminus of GLUT4 contains sequences essential for the proper trafficking and localization of GLUT4 (19, 24–26), and it is therefore possible that the GFP interferes with the function of these signals. Our previous assessment that HA-GLUT4-GFP traffics like endogenous GLUT4 was based on comparisons of insulin-stimulated translocation (5–8). To more completely assess possible effects of the carboxyl-terminal GFP, we examined the behavior of the HA-GLUT4 construct by electroporation (27).

To analyze the trafficking of HA-GLUT4 transiently expressed by electroporation, the total expression of the construct was measured by incubating cells for 2 h at 37 °C in medium with insulin and Cy3-HA-11, followed by a 2-h, 37 °C incubation in medium without insulin to return to basal conditions (supplemental Fig. 2). This method of measuring total expression was validated by comparing the translocation of HA-GLUT4-GFP using either GFP or Cy3-HA-11 as a measure of total HA-GLUT4-GFP expression (supplemental Fig. 2). Basal retention and insulin-stimulated translocation were similar whether the Cy3 or GFP fluorescence was used, indicating that prelabeling GLUT4 with Cy3-HA-11 at the start of the experiment does not perturb HA-GLUT4-GFP trafficking, and therefore it can be used to normalize for exogenous GLUT4 expression. Furthermore, translocation of HA-GLUT4-GFP prebound by HA-11 is within the range observed for translocation of HA-GLUT4-GFP unbound by HA-11, confirming that antibody binding does not detectably alter HA-GLUT4-GFP trafficking (5, 6, 8).

Next, we compared the translocation of HA-GLUT4 to HA-GLUT4-GFP as a means of assessing the effect of the carboxyl-terminal GFP. Cells, preincubated with Cy3-HA-11 at 37 °C and then reacted with Cy5-goat anti-mouse antibody; therefore, a rise in the Cy5/Cy3 ratio over time reflects the exocytosis of HA-GLUT4 to the cell surface, with this ratio reaching a plateau when all of the cycling HA-GLUT4 has equilibrated with the plasma membrane (8, 28). The plateau level reached after about 6 h of incubation in basal conditions was similar to the plateau reached after 1 h in the presence of insulin, providing evidence that the basal cycling pool of HA-GLUT4 is similar in size to the pool recruited by insulin (Fig. 1B). The half-time to reach the plateau in the basal state was similar to what was previously seen for HA-GLUT4-GFP (8). These data are consistent with our previous experiments demonstrating that GLUT4 effluxes slowly in the basal state and quickly in the insulin state, and they demonstrate that the carboxyl-terminal GFP of the HA-GLUT4-GFP reporter does not affect the exocytosis kinetics of GLUT4.

To determine whether the method of expression of the reporter had an effect on the behavior of GLUT4 in basal conditions, we studied the behavior of HA-GLUT4 expressing retroviral infection. The average expression level of total GLUT4 in cells infected with a retrovirus coding for HA-GLUT4 was less than twice that of endogenous GLUT4 in control cells (Fig. 3A).

The efflux kinetics of HA-GLUT4 in the basal and the insulin-stimulated states were measured using a modification of the HA-11 antibody uptake assay discussed above. Day 5 adipocytes expressing HA-GLUT4 were incubated at 37 °C for various times in DMEMBB containing HA-11 (without prior pretreatment with insulin). At each time point, the amount of cell-associated HA-11 was determined by indirect immunofluorescence. The expression level of HA-GLUT4 within an individ-
first determined that 0.5 nM insulin promoted an approximate half-
lation of Exocytosis and Submaximal Inhibition of Endocytosis
as we have previously reported for HA-GLUT4-GFP expressed by
in insulin-stimulated conditions, only with significantly slower kinetics.
Therefore, HA-GLUT4 expressed by retroviral infection is in equilib-
ized to the insulin plateau value within each experiment. The data are normalized
to the insulin plateau value within each experiment. The data are the average of
seven experiments ± S.E. C, the left panel shows the GLUT4 expression pattern in control
cells not infected with HA-GLUT4, and the center and right panels show the patterns of
HA.11 taken up during the indicated incubation visualized by indirect immunofluores-
cence. Representative fields collected with a 40× objective are shown.

FIGURE 3. Efflux of HA-GLUT4 expressed by retroviral infection. A, quantification of
total GLUT4 in HA-GLUT4-infected cells compared with uninfected (control) cells
expressing only endogenous GLUT4, determined by indirect immunofluorescence with
anti-GLUT4 antibody. The average GLUT4 expression/cell ± S.E., expressed as arbitrary
fluorescence units (A.F.U.), from at least 100 cells/condition are shown. B, quantification of
HA.11 uptake in cells expressing HA-GLUT4 by retroviral infection. The data are normal-
ized to the insulin plateau value within each experiment. The data are the average of
seven experiments ± S.E. C, the left panel shows the GLUT4 expression pattern in control
cells not infected with HA-GLUT4, and the center and right panels show the patterns of
HA.11 taken up during the indicated incubation visualized by indirect immunofluores-
cence. Representative fields collected with a 40× objective are shown.

uval cell was not accounted for, since the average expression level did not
vary significantly from dish to dish within an experiment. As was the
case for HA-GLUT4 expressed by electroporation (Fig. 2B), in basal
conditions HA-GLUT4 expressed by retroviral expression cycles to the
cell surface with a halftime of about 3–4 h (Fig. 3B). The same plateau
level of labeling was reached in the basal as in the insulin-stimulated
state. After 10 h of uptake, the HA.11 is localized to the perinuclear
region of the cell in a pattern that corresponds to the pattern of endog-
ous GLUT4 staining in untransfected 3T3-L1 adipocytes (Fig. 3C).
Therefore, HA-GLUT4 expressed by retroviral infection is in equilib-
rium with the plasma membrane in the basal state to the same extent as
in insulin-stimulated conditions, only with significantly slower kinetics,
as we have previously reported for HA-GLUT4-GFP expressed by
electroporation.

Intermediate GLUT4 Redistribution Is Achieved by Submaximal Stimula-
tion of Exocytosis and Submaximal Inhibition of Endocytosis—To analyze
the behavior of GLUT4 in submaximal insulin concentrations, we first determined that 0.5 nM insulin promoted an approximate half-
maximal redistribution of GLUT4 to the plasma membrane (Fig.
4A). A similar half-maximal insulin concentration for stimulated glucose uptake in 3T3-L1 adipocytes has been reported (29).

The average intermediate response of the population of cells could result from individual cells responding to an intermediate extent or
heterogeneity in the population of cells, with some cells responding fully
and others not at all. To address this question, the results from the experiments in Fig. 4A were analyzed on a cell-by-cell basis. In the
majority of cells treated with 0.5 nM insulin, the amount of GLUT4 on
the surface was intermediate between basal and the 200 nM, indicating
that the partial translocation is due to individual cells responding to an
intermediate extent (Fig. 4B).

We next measured the time required to achieve a new surface steady-
state level of HA-GLUT4-GFP in 0.5 nM insulin. The time required to
reach the plateau level in surface HA-GLUT4-GFP was longer in 0.5 nM
than in 200 nM insulin (Fig. 4C). This difference is unlikely to be due
totally to a delay in the signaling from the insulin receptor, since the
time of the initiation of the signaling cascade, 1.5 min for IRS in 10
and 100 nM insulin (30–32), is fast compared with the time needed to
reach the steady-state distribution of GLUT4. Additionally, because of
the amplification of the signal, the sensitivity to insulin increases for the
molecules further downstream in the signaling cascade (30). The
observed slower rate of response to 0.5 nM insulin may instead be the
consequence of slower overall kinetics of GLUT4-containing vesicles to
the plasma membrane. If the rate of GLUT4 efflux is slower, then it will
take longer to accumulate enough GLUT4 at the plasma membrane to
reach the new surface population level.

We next measured the steady-state efflux rate of GLUT4 in the pres-
ence of 0.5 nM insulin. The cells were preincubated for 1 h, at 37 °C, with
Both the 200 and 0.5 nM insulin curves fit well to a single exponential, rate constant. The internalization parameters of individual experiments were normally distributed around a single mean. The internalization parameter, which is the slope of a plot of the fraction of surface intracellular pool recruited to the plasma membrane in 200 nM insulin vs. time, was determined by indirect immunofluorescence using Cy3-goat anti-mouse antibody. Data are normalized to the 200 nM plateau level. The data are fit to a single exponential described in the legend to Fig. 2. Both the 200 and 0.5 nM data are well fit by a single exponential; $r = 0.98$ and 0.97, respectively. The $K_{\text{off}}$ measured for 0.5 nM insulin was about half of that measured for 200 nM insulin, 0.019 ± 0.014 min$^{-1}$ versus 0.05 ± 0.014 min$^{-1}$, respectively. The data are the average of five experiments ± S.E.

A. quantification of HA.11 uptake in the presence of protease inhibitors and 0.5 or 200 nM insulin in cells electroporated with HA-GLUT4-GFP. Cells were prestimulated with the appropriate concentration of insulin for 1 h prior to the incubation with HA.11. The amount of cell-associated HA.11 after each time point was determined by indirect immunofluorescence using Cy3-goat anti-mouse antibody. Data are normalized to the 200 nM plateau level. The data are fit to a single exponential described in the legend to Fig. 2. Both the 200 and 0.5 nM data are well fit by a single exponential; $r = 0.98 \text{ and } 0.97$, respectively. The $K_{\text{off}}$ measured for 0.5 nM insulin was about half of that measured for 200 nM insulin, 0.02 ± 0.005 min$^{-1}$ versus 0.05 ± 0.014 min$^{-1}$, respectively. The data are the average of five experiments ± S.E.

B. quantification of GLUT4 recycling in the presence of submaximal insulin. GLUT4 was cycling between the intracellular and plasma membrane. The data are the average of five experiments ± S.E.

C. quantification of GLUT4 recycling in the presence of submaximal insulin. GLUT4 was cycling between the intracellular and plasma membrane. The data are the average of five experiments ± S.E.

insulin to ensure that GLUT4 trafficking between the interior and surface of cells was at steady state. The halftime to reach the plateau was longer in 0.5 nM insulin than in 200 nM insulin, establishing that the overall exocytosis kinetics in submaximal insulin are slower (Fig. 5A). Both the 200 and 0.5 nM insulin curves fit well to a single exponential, indicating that GLUT4 is cycling as a single kinetic pool under both conditions. The plateau level reached was the same in 0.5 nM insulin as in 200 nM insulin, demonstrating that, as in basal conditions, the entire intracellular pool recruited to the plasma membrane in 200 nM insulin was recruited in submaximal insulin conditions, just with different kinetics. We conclude that an intermediate steady-state surface-to-total GLUT4 distribution is the result of the entire pool of GLUT4 cycling to the plasma membrane at a net intermediate rate.

Another parameter of GLUT4 trafficking regulated by insulin is its internalization (7, 12, 33). Insulin inhibition of GLUT4 internalization contributes to the net accumulation of GLUT4 on the cell surface. We found that GLUT4 internalization was inhibited by about 70% in 200 nM insulin and about 50% in 0.5 nM insulin (Fig. 5B). Thus, like exocytosis, submaximal insulin mediates an intermediate effect on GLUT4 internalization kinetics, indicating that regulation of endocytosis and exocytosis is graded rather than binary.

To determine whether the effect of insulin on internalization is specific to GLUT4, and not a general perturbation of membrane trafficking, we measured the effect of insulin on TR internalization. We found that TR internalization was not slowed by the presence of insulin. There was actually a slight increase in the internalization rate in the presence of 200 nM insulin (Fig. 5C). This may be due to a general increase in membrane flux in the presence of insulin (34). The effect of insulin on slowing internalization is therefore specific to GLUT4.

**DISCUSSION**

The results of this study demonstrate that intracellular GLUT4 is in equilibrium with the plasma membrane in basal adipocytes and that insulin stimulates this exchange rate. Our data show that neither attachment of GFP on the carboxyl terminus of GLUT4 nor the means of transfection, electroporation or retroviral infection, detectably influence the trafficking behavior of the GLUT4 reporters. These data provide additional support for the dynamic, bipartite retention model (8).

The dynamic, bipartite mechanism of GLUT4 retention proposed here is not unlike other retention mechanisms used in the cell. Retention of ER- and Golgi-resident proteins is achieved by repeated cycles of release and retrieval (35, 36). Even in the tightly regulated system of synaptic vesicle release, a basal current is maintained during the unstimulated state (37). The dynamic bipartite nature of such systems may facilitate the ability of the cell to respond rapidly to acute environmental changes. This retention mechanism might be expected to be analogous to a rheostat. By having a resistor on the system that can change the “current” allowed to flow through the system, without ever cutting off the flow entirely, the system would remain in continual motion and thus be more sensitive to changes. Perhaps it is more efficient for a cell to make kinetic changes in an already mobile system than to continually stop and start the system.

To further our understanding of insulin action, we examined GLUT4 trafficking at submaximal concentrations of insulin. Such studies can provide a glimpse of the transition state between the basal and maximal insulin states and thus help us to build a better model of the mechanism of insulin action. Additionally, most studies of GLUT4 trafficking in 3T3-L1 adipocytes have made comparisons between basal (no insulin) and insulin concentrations greater than 100 nM. These experimental conditions probably do not reflect normal physiologic conditions (16, 17).

We found that the cellular distribution of GLUT4 in submaximal insulin conditions, like in basal, and maximal insulin conditions is established by changes in the rate of exchange of GLUT4 between the intracellular storage pool and the plasma membrane. The mechanism of insulin action is dependent on the modulation of kinetic rates of GLUT4 trafficking at multiple steps of the pathway. These results are consistent with a gradated transition from the basal to the maximal insulin stimulated state; as the concentration of insulin is increased, there is a proportional increase in the efflux rate and a decrease in the internalization rate. The submaximal insulin studies establish that the models for GLUT4 trafficking developed using extreme insulin concentrations (no
insulin and greater than 100 nM insulin) are applicable to the behavior of GLUT4 in an insulin concentration more in the physiological range, 0.5 nM.

The consequence of this bipartite mechanism of regulation of GLUT4 distribution can be better understood by examining a three-dimensional model of the calculated fraction of GLUT4 on the cell surface as a function of varying exocytosis and endocytosis constants measured for GLUT4 in adipocytes stimulated with greater than 100 nM insulin (maximal stimulation). Those values were derived from this current study (O. J. Martin, A. Lee, and T. E. McGraw, unpublished data) and previous published studies (7, 8). To the right of the graph is the plasma membrane GLUT4 color code. The plot was generated using SigmaPlot.

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