Kinetic Evidence That Glut4 Follows Different Endocytic Pathways than the Receptors for Transferrin and \(\alpha_2\)-Macroglobulin*

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Insulin regulates glucose uptake through effects on the trafficking of the glucose transporter Glut4. To investigate the degree of overlap between Glut4 and the general endocytic pathways, the kinetics of trafficking of Glut4 and the receptors for transferrin (Tf) and \(\alpha_2\)-macroglobulin (\(\alpha_2\)-M; LRP-1) were compared using quantitative flow cytometric assays. Insulin increased the exocytic rate constant \((k_{\text{ex}})\) for both Glut4 and Tf. However, the \(k_{\text{ex}}\) of Glut4 was 5–15 times slower than Tf in both basal and insulin-stimulated cells. The endocytic rate constant \((k_{\text{en}})\) of Glut4 was also five times slower than Tf. Insulin did not affect the \(k_{\text{en}}\) of either protein. In basal cells, the \(k_{\text{en}}\) for \(\alpha_2\)-M/LRP-1 was similar to Glut4 but 5-fold slower than Tf. Insulin increased \(k_{\text{en}}\) for \(\alpha_2\)-M/LRP-1 by 30%. In contrast, the \(k_{\text{en}}\) for LRP-1 was five times faster than Glut4 in basal cells, and insulin did not increase this rate constant. Thus, although there is overlap in the protein machineries/compartments utilized, the differences in trafficking kinetics indicate that Glut4, the Tf receptor, and LRP-1 are differentially processed both within the cell and at the plasma membrane. It has been reported that insulin decreases the \(k_{\text{en}}\) of Glut4 in adipocytes. However, the effect of exocytosis on the “internalization” assays was not considered. Because it is counterintuitive, the effect of exocytosis on these assays is often overlooked in endocytosis studies. Using mathematical modeling and simulation, we show that the reported decrease in Glut4 \(k_{\text{en}}\) can be entirely accounted for by the well-established increase in Glut4 \(k_{\text{ex}}\).

Glucose transporter 4 (Glut4) is the major insulin-sensitive glucose transporter expressed in muscle and adipose tissues (1). In unstimulated basal adipocytes, Glut4 is largely sequestered away from the plasma membrane in intracellular storage compartments (Glut4 storage vesicles or GSVs).\(^3\) Insulin stimulation leads to the redistribution of Glut4 from GSVs to the plasma membrane within minutes. Regulation of the translocation and insertion of Glut4 into the plasma membrane in response to insulin is critical for the proper disposal of postprandial excess glucose.

Equally important is the other arm of Glut4 trafficking, Glut4 endocytosis. The timely removal of Glut4 from the plasma membrane prevents hypoglycemia and ensures the internalized Glut4 is directed to its intracellular storage destination. The efficient removal of glucose from the blood and the subsequent decline in insulin signaling starts the shift in Glut4 trafficking. Once insulin signaling starts to diminish, Glut4 endocytosis becomes the predominant Glut4 trafficking arm/pathway until greater than 90% of Glut4 is removed from the plasma membrane (2, 3). This dynamic exchange of Glut4 between the intracellular compartments and the plasma membrane is what keeps blood sugar levels within an optimal range under normal physiological conditions. Strict regulation of Glut4 exocytosis and endocytosis is crucial to the homeostasis of whole body glucose. Therefore, it is critical to characterize both arms of Glut4 trafficking to fully understand how specific steps of Glut4 trafficking (endocytosis/exocytosis) are regulated by insulin.

Glut4 trafficking involves the general endocytic pathway as well as specialized pathways unique for its function (4–8). There are two intracellular pools of Glut4 in basal cells as follows: a cycling pool (10–25%) that is continuously exchanging with the Glut4 at the plasma membrane, and a “static” GSV pool (75–90%) that is either not actively exchanging with the plasma membrane pool or is exchanging very slowly.\(^4\) Insulin has at least two effects on Glut4 trafficking as follows: 1) insulin releases Glut4 from the GSVs, increasing the actively cycling pool 3–10-fold; and 2) insulin increases \(k_{\text{en}}\), the rate constant for delivery of Glut4 from the actively cycling pool to the plasma membrane, 3–7-fold (2, 3, 9–12). The detailed itinerary of Glut4 from the plasma membrane to the storage compartments and back to the cell surface has yet to be fully characterized.

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3 The abbreviations used are: GSV, Glut4 storage vesicle; \(\alpha_2\)-M, \(\alpha_2\)-macroglobulin; In/Sur, internal/surface; LSM, low serum media; Lyi, LY294002; MFR, mean fluorescence ratio; Tf, transferrin; TfR, Tf receptor; MFI, mean fluorescence intensity.

4 It is well established that there are two pools of Glut4, one in the general endocytic pathway and one in the specialized storage compartments, GSVs. It is not well understood how Glut4 exchanges between these pools. In some experimental systems, including ours, there is very little or no exchange between these pools in basal cells (3, 10). In others, there is a slow but measurable exchange (12, 14). We believe that this difference simply depends on how tightly the GSVs are regulated under different experimental cell culture conditions and is not due to qualitative differences in the mechanism of regulation. Under our culture conditions, effects on the sequestration of Glut4 in GSVs can be distinguished from effects on trafficking kinetics. In other systems, the slow rate of release from the GSVs \((t_{1/2} = 4 \text{ h})\) masks the kinetics of a small, more rapidly cycling pool \((t_{1/2} = 1 \text{ h})\). Under these conditions, release of Glut4 from slowly cycling GSVs into the rapidly cycling pool would manifest as an increase in the rate constant for exocytosis and not an increase in the size of the cycling pool.
The Glut4 inside of the cell is co-localized with markers of both early endosomes (the transferrin (Tf) receptor) and late endosomes (low density lipoprotein receptor-related protein or LRP-1, the α-2-M receptor expressed in adipocytes) (13–15). It is not known whether Glut4 preferentially traffics within early or late endosomes or to what extent Glut4 trafficking overlaps with these pathways. It is clear that although they are co-localized after internalization, Glut4 is sorted away from the Tf receptor during its intracellular cycling (16–18). This occurs before the Glut4 is packaged into the GSVs (19, 20). In contrast, proteomic analysis of the GSVs showed extensive co-localization of LRP-1 with Glut4 in the storage compartments (15). These observations suggest that Glut4 may be trafficked with LRP-1 within the late endosomal pathway and that GSVs may be a specialization of this pathway.

Endosomal ligand sorting begins at the plasma membrane. Although the Tf receptor is recruited into clathrin-coated pits through interaction with the ubiquitous adaptor protein AP2, late endosomal markers such as the receptors for LDL and EGF are recruited into clathrin-coated pits via unique/specific adaptor proteins (21–25). A number of studies have indicated that Glut4 is also endocytosed via a different mechanism than the Tf receptor. For example, although endocytosis of the Tf receptor is significantly inhibited by knockdown of AP2, endocytosis of Glut4 is much less affected (26). Additional studies suggest that Glut4 is internalized through both clathrin-mediated endocytosis and clathrin-independent endocytic pathways (reviewed in Ref. 27). In general, differences in the sensitivity of the internalization of Glut4 and the Tf receptor to different treatments have been cited as evidence for differences in the pathways of internalization. However, interpretation of the previously published data is made difficult by the fact that the effect of changes in the exocytosis of Glut4 on the “internalization” assays was generally not considered.

We hypothesized that the actively cycling Glut4 may simply be carried as cargo with other proteins in the endocytic pathway and that it is only the sequestered GSV pool that is trafficked in a specialized way. To test this hypothesis, we compared the rates of trafficking of Glut4 and two endocytic markers, the Tf receptor and the receptor for α-2-M, LRP-1. If the cycling Glut4 is simply trafficking as an endocytic protein, then the kinetics of internalization and exocytosis should be the same for the cycling Glut4 as for the other endocytic markers. However, differences in the intrinsic rate constants of endocytosis or exocytosis would demonstrate that there are differences in the mechanisms of trafficking of Glut4 and other endocytic proteins, independent of whether there is overlap in the protein machineries or compartments that are utilized. Therefore, we compared the kinetics of trafficking of Glut4, the Tf receptor, and α-2-M/LRP-1 in 3T3-L1 adipocytes under identical experimental conditions to examine the following: 1) the relative rate constants of internalization and cycling of these markers, and 2) the effect of insulin on their rate constants.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Viral Infection**

3T3-L1 fibroblasts were differentiated into adipocytes as described previously (3). Briefly, fibroblasts were plated into 96-well plates. After reaching confluency, they were incubated for 3 days in differentiation medium (DMEM complete media, high glucose DMEM supplemented with 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin), 10% FBS (Hyclone), 5 μM troglitazone (EMD Chemicals), dexamethasone, isomethylbutylnxanthine, and insulin), 3 days in insulin medium (DMEM complete media, 10% FBS, troglitazone, dexamethasone, and insulin), and then 4–5 days in DMEM complete media, 10% FBS. Cells were used 10–13 days after the initiation of differentiation. For some experiments, fibroblasts were transduced with lentivirus expressing the HA-Glut4/GFP reporter construct before being differentiated, as described previously (3).

**Antibodies and Reagents**

HA.11 anti-HA monoclonal antibody was purchased from Covance as ascites and purified using a 1–ml HiTrap Protein-A FF column (GE Healthcare) as described previously (3). The purified antibody was then labeled with Alexa647 using a monoclonal antibody labeling kit (Invitrogen) according to the manufacturer’s recommended protocol (antibody was incubated with dye for 2 h at room temperature and then overnight at 4 °C). After incubation, free dye was removed by desalting twice into PBS using 2-ml Zeba desalting spin columns (7000 MWCO; ThermoScientific). Antibody concentrations and labeling efficiency were determined by absorbance spectroscopy as described previously (3). α-2-M was purchased from Assaypro. To remove glycine before labeling, α-2-M was desalted into PBS using a 5-ml HiTrap desalting column (GE Healthcare). α-2-M was then labeled with Alexa647 with an antibody labeling kit as described above. The α-2-M was activated by adding methylamine (28). To minimize dilution from multiple runs through the desalting columns, the labeled α-2-M was activated by adding methylamine directly to the reaction mix. Quenched dye and excess methylamine were removed by desalting into PBS before use in assays. Iron-loaded (holo) Tf conjugated with Alexa647 was purchased from Invitrogen. Unlabeled holo-Tf and chloroquine were purchased from Sigma.

**Reversible Labeling of Anti-HA Monoclonal Antibody**

Antibody was purified as described above and concentrated to 2–3 mg/ml using Microcentrifugal concentrators (30,000 MWCO; Millipore). Concentrated antibody was desalted into modification buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) using two 0.5-ml Zeba columns in series. Antibody was modified with a 10-fold molar excess of succinimidyl disulfide 4-formylbenzoate (S-SS-4FB, Solulink) according to the manufacturer’s instructions. This converts amines into disulfide-linked aromatic aldehydes. Modified antibody was desalted into conjugation buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 6.0). Protein concentration was determined via BCA assay, and the extent of modification was determined using 2-hydrazinopyridine colorimetric assay according to the manufacturer’s protocol (Solulink). Modified antibody was then incubated with Alexa647 hydrazide (Invitrogen) at a 20-fold excess over modified residues (3 h at room temperature and then overnight at 4 °C). Conjugated
antibody was desalted into PBS, and protein concentration was determined using BCA before using in assays.

**Steady-state Anti-HA Uptake**

3T3-L1 adipocytes expressing HA-Glut4/GFP were preincubated for 2 h in low serum media (LSM; DMEM complete media supplemented with 0.5% FBS). For insulin-stimulated uptake, the LSM was replaced with LSM-containing insulin for the final 30 min of incubation (100 nM unless otherwise indicated). At various times, the incubation media were removed and replaced with LSM containing 50 μg/ml Alexa647-anti-HA. At the end of the incubation, the plate of cells was placed on ice, washed with ice-cold PBS, collagenase-digested, and analyzed by flow cytometry as described previously (3). The data were fitted by nonlinear regression for a single exponential association as shown in Equation 1,

$$Y = Y_{\text{min}} + (Y_{\text{max}} - Y_{\text{min}}) \times (1 - \exp(-k_{\text{obs}} \times t)) \quad \text{(Eq. 1)}$$

where $k_{\text{obs}}$ is the observed relaxation rate constant. In the uptake experiments, $Y_{\text{max}}$ is the cycling pool size, and $k_{\text{obs}} = k_{\text{en}}$, the rate constant for exocytosis (insertion of Glut4 into the plasma membrane).

**Glut4 Transition Kinetics**

3T3-L1 adipocytes expressing HA-Glut4/GFP were serum-starved as described above. For the basal to insulin transitions, the LSM was replaced with LSM containing 100 nm insulin, and incubation was continued for increasing amounts of time. For the insulin + inhibitor transitions, LSM was replaced with LSM containing 100 nm insulin for the last 30 min of starvation; then 50 nm LY294002 (EMD Chemicals) was added and incubation continued for increasing amounts of time. After the time course was completed, the cells were placed on ice, and surface Glut4 was labeled with Alexa647-anti-HA. Cells were then analyzed by flow cytometry. The data were fitted by single exponential equations (either association or decay). In the basal to insulin transition experiments, $Y_{\text{max}}$ is the amount of Glut4 in the plasma membrane after the new equilibrium level is reached, and $k_{\text{obs}} = k_{\text{en}} + k_{\text{ex}}$, where $k_{\text{en}}$ is the rate constant for endocytosis, internalization of Glut4 (29). In the insulin + inhibitor transition experiments, $k_{\text{obs}} \approx k_{\text{en}} k_{\text{ex}}$ (also equals $k_{\text{en}} + k_{\text{ex}}$ but $k_{\text{ex}}$ is very low after the addition of PI3K inhibitors; Fig. 1) (30, 31).

**α-2-Macroglobulin Uptake and Surface Labeling**

3T3-L1 adipocytes were serum-starved in serum-free DMEM for 2 h and then incubated with or without insulin (100 nm) for 30 min. Cells were then incubated with 4 μg/ml Alexa647 α-2-M in serum-free DMEM for the indicated times at 37 °C. At the end of the time course, cells were placed on ice, and additional cells on the same plate were incubated for 90 min with Alexa647 α-2-M to label surface receptors. To prevent loss of surface α-2-M during subsequent incubations, cells were warmed to 37 °C for 10 min to internalize a single surface cohort of label. Cells were analyzed by flow cytometry. The data were fitted to linear equations. To measure the internalization rate constants, the internal to surface (In/Sur) ratio for each time point was determined. The slope of In/Sur versus time plot equals $k_{\text{en}}$, (32).

**Transferrin Uptake and Efflux**

For Tf uptake experiments, 3T3-L1 adipocytes were preincubated for 2 h in LSM. For insulin-stimulated uptake, the LSM was replaced with LSM containing insulin for the final 30 min of incubation. At various times the incubation media were removed and replaced with LSM with or without insulin containing Alexa647-conjugated, iron-loaded (holo) Tf (5 μg/ml), and incubation was continued at 37 °C. The plates were then placed on ice and washed with ice-cold LSM containing unlabeled holo-Tf (500 μg/ml). In a duplicate set of samples, 100-fold excess unlabeled holo-Tf was added at the end of the initial incubation, and incubation was continued for 70 min. For the Tf efflux assay, cells were labeled with Alexa647-conjugated holo-Tf for 30 min at 37 °C. The cells were rapidly washed and then incubated at 37 °C in LSM with excess unlabeled holo-Tf. Incubation in media with excess unlabeled holo-Tf was sufficient to remove all surface Tf. Cells were analyzed by flow cytometry. The data were fitted by equations for a single exponential decay. In these experiments $k_{\text{obs}} = k_{\text{ex}}$ (33, 34).

**Determination of Partition Coefficients (P)**

Glut4—3T3-L1 adipocytes expressing HA-Glut4/GFP were preincubated in LSM and then stimulated with insulin (0–100 nm) as indicated) for 30 min at 37 °C. After insulin stimulation, the cycling pool of Glut4 was labeled by incubating cells in media supplemented with 20 μg/ml reversibly labeled antibody (Alexa647-SS-anti-HA) and insulin for 4 h at 37 °C. After this incubation, the plate was chilled on ice, and antibody was added to additional cells on the same plate to label surface Glut4 (1 h of incubation). The cells were washed (200 μl, three times for 15 min) with either freshly prepared cleavage buffer (0.5× PBS supplemented with 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 100 mM glutathione, pH 8.6) or control buffer (0.5× PBS supplemented with 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 50 mM NaCl, pH 8.6, plus additional NaCl to match osmolarity of NaOH added to cleavage buffer for pH adjustment) (35). All cells were then rinsed with PBS to remove glutathione, collagenase-digested, and analyzed by flow cytometry. Efficiency of cleavage was determined by the ratio of (surface-labeled uncleaved – surface-labeled cleaved)/(surface-labeled uncleaved) and was consistently greater than 95%. The partition coefficient (P) is a ratio of the amount of Glut4 present in the plasma membrane to the amount of Glut4 cycling inside the cell. It is important to note that P does not include the noncycling Glut4 present in static GSVs at submaximal insulin concentrations. For this experiment, surface Glut4 is equal to the total Glut4 labeled at 37 °C minus the amount inside resistant to glutathione cleavage. Thus, P = (plasma membrane Glut4)/(intracellular cycling Glut4) = (labeled uncleaved – labeled cleaved)/(labeled cleaved).

**α-2-M**—To estimate the partition coefficient of LRP-1, cells were treated with 60 μM chloroquine to inhibit dissociation of α-2-M from its receptor (36, 37) and then incubated as described above with Alexa647 α-2-M at 37 °C for the indicated times. Cells were placed on ice, and additional cells were surface-labeled as described above. Any α-2-M remaining on the
cell surface at the end of the incubations was rapidly lost after washing the cells and subsequent incubation at 4 °C. The data were fitted by single exponential associations, where $Y_{\text{max}}$ is the amount of intracellular LRP-1.

Flow Cytometry, Gating, and Data Analysis

Cells were analyzed by flow cytometry as described previously (3). Briefly, cells were collagenase-digested and gently resuspended in a final volume of 200 μl of PBS. Suspended cells were filtered through a 100-μm cell strainer and analyzed by flow cytometry on a FACScan cytometer (BD Biosciences). Adipocytes were gated from fibroblasts and cellular debris using FlowJo software (Tree Star) as described previously. For anti-HA experiments, background fluorescence was corrected by subtracting the FL4 and FL1 values of uninfected cells from those of infected cells. Mean fluorescence ratios (MFRs) were then determined by the ratio of background-corrected FL4 (antibody labeling) to background-corrected FL1 (total HA-Glut4/GFP reporter). For the α-2-M and TF experiments, the geometric mean of FL4 was corrected for background auto-fluorescence and used to calculate rate constants.

RESULTS

The rate constants for insertion of Glut4 into the plasma membrane ($k_{\text{ex, rate constant}}$) were measured directly in an antibody uptake assay (Fig. 1) (3). In these experiments, cells expressing an exofacial epitope-tagged HA-Glut4/GFP reporter construct were incubated with Alexa647-conjugated anti-HA antibody at 37 °C (at 50 μg/ml, a saturating concentration), and the accumulation of fluorescent antibody was measured at increasing times. Within the resolution of the experimental data, the Glut4 at the cell surface was instantaneously labeled. Therefore, the rate of accumulation of additional labeled antibody was completely dependent on the rate of delivery of the unlabeled intracellular Glut4 to the cell surface. Labeling followed a single exponential, and the observed rate constant equaled $k_{\text{ex}}$. Our measured exocytic rate constants for Glut4 were as follows: $k_{\text{ex, insulin}} = 0.008 ± 0.003$ min$^{-1}$; $k_{\text{ex, basal}} = 0.04 ± 0.004$ min$^{-1}$ (Fig. 1A and Table 1). Insulin also increased the cycling pool size by 3-fold ($Y_{\text{max, insulin}} = 0.33 ± 0.05 × Y_{\text{max, basal}}$). Both $k_{\text{ex}}$ and $Y_{\text{max}}$ showed incremental dose-dependent increases in response to insulin (Fig. 1B) (3, 10).

Unlike anti-HA uptake, the kinetics of TF uptake in 3T3-L1 adipocytes were complex and could not be fit with a single exponential (Fig. 2A). In these experiments, cells were incubated with Alexa647-conjugated iron-loaded (holo) TF at 37 °C (5 μg/ml, a saturating concentration) and accumulation of fluorescent TF measured after increasing times. In a duplicate set of samples, 100-fold excess unlabeled holo-TF was added at the end of the initial incubation, and incubation was continued for 70 min. When the TF receptor was internalized, the iron dissociated from the TF, but the iron-depleted (apo) TF remained attached to its receptor and returned to the cells surface. When the apo TF recycles to the plasma membrane, it dissociated from its receptor and was replaced by holo-TF, which has a 5-fold higher affinity for the TF receptor at neutral pH (38–40). Thus, labeled TF traffics with its receptor through a single complete cycle. Therefore, TF labeling was expected to follow a single exponential-like anti-HA uptake. However, TF labeling was biphasic, with a rapid exponential filling of a pool that could be completely chased from the cells and a slower accumulation of TF that could not be chased from the cells after a 70-min incubation with unlabeled TF. This indicates either

FIGURE 1. Determination of $k_{\text{ex}}$ for Glut4 (anti-HA uptake). A, anti-HA uptake. 3T3-L1 adipocytes expressing HA-Glut4/GFP treated with [□] or without [■] insulin (100 nM, 30 min) or with LY294002 (50 nM, 45 min) prior to insulin ([)], LY1 + insulin) were incubated with Alexa647-conjugated anti-HA antibody (50 μg/ml) for 10–240 min at 37 °C. Samples were analyzed by flow cytometry as described previously (3). Data points are the average MFR of Alexa647/GFP ± S.E. (S.E.; n = 3 replicate experiments). Line, single exponential fits. The data were standardized to the MFRmax of the 100 nM insulin samples. $k_{\text{ex, insulin}} = 0.008 ± 0.003$ min$^{-1}$; $k_{\text{ex, basal}} = 0.04 ± 0.004$ min$^{-1}$; $k_{\text{ex, LY1 + insulin}} = 0.004 ± 0.003$; insulin stimulates dose-dependent increases in both the rate constant of Glut4 exocytosis ($k_{\text{ex}}$) and the cycling pool size ($Y_{\text{max}}$). 3T3-L1 adipocytes expressing HA-Glut4/GFP were treated with the indicated concentrations of insulin (0–100 nM insulin, 30 min), then incubated with Alexa647 anti-HA for the indicated times, and analyzed by flow cytometry.

TABLE 1

Summary of rate constants for exocytosis ($k_{\text{ex}}$) and endocytosis ($k_{\text{en}}$) and $Y_{\text{max}}$

|          | $k_{\text{ex}}$ (min$^{-1}$) | $k_{\text{en}}$ (min$^{-1}$) | $Y_{\text{max}}$ |
|----------|-----------------------------|-----------------------------|-----------------|
|          | Basal       | Insulin     | Basal       | Insulin     |Basal       | Insulin     |
| Glut4    | 0.008 ± 0.003 | 0.04 ± 0.004 | 0.12 ± 0.01 | 0.12 ± 0.013 | 0.1–0.2 | 1.0 |
| TIR      | 0.12 ± 0.011 | 0.20 ± 0.015 | 0.60 ± 0.05 | 0.60 ± 0.04 | 1.0 | 1.0 |
| LRP-1    | 0.04 ± 0.008 | 0.05 ± 0.001 | 0.09 ± 0.004 | 0.12 ± 0.005 | 0.5–0.6 | 1.0 |
that there is more than one pool of cycling Tf receptor (one cycling very fast, and the other very slowly) or that a small fraction of the Tf dissociates from the receptor within endosomes with each cycle and accumulates in the cells instead of recycling.

Because of the complexity of the uptake kinetics, efflux assays were done to measure the exocytosis kinetics of the Tf receptor (Fig. 2B). In these experiments, cells were incubated with Alexa647-holo-Tf at 37 °C for a short time to label predominantly the pool of fast cycling receptors, and then 100-fold excess unlabeled holo-Tf was added and incubation continued for the indicated times. The loss of labeled Tf with time was measured. To avoid accumulation of Tf in noncycling/slowly cycling compartments, cells were labeled for 30 min or less. The loss of labeled Tf with time was examined for increasing times. Data points are the average mean fluorescence intensities (MFI) Alexa647 fluorescence intensities (MFI) Alexa647 continued for increasing times (Fig. 2). Tf uptake shows complex kinetics. Uninfected 3T3-L1 adipocytes were incubated with unlabeled holo-Tf (500 μg/ml) at 37 °C for the indicated times (basal). Specific uptake is as follows: Pulse-Chase (Fig. 2B, Tf efflux). Uninfected 3T3-L1 adipocytes were incubated with or without insulin, then labeled with Alexa647 holo Transferrin (Tf; 5 μg/ml) at 37 °C for 30 min. 100-fold excess unlabeled holo-Tf (500 μg/ml) was added, and incubation continued for increasing times. Data points are the average mean fluorescence intensities (MFI) Alexa647 ± S.E. (n = 6). The data were standardized to the initial MFI for each condition (initial MFIinsulin was 90% of the initial MFIbasal). Line, single exponential fits. kex basal = 0.12 ± .011 min⁻¹; kex insulin = 0.20 ± .015 min⁻¹.

The rate constants for internalization of Tf from the plasma membrane (kex indicates endocytic rate constants) were calculated from the measured partition coefficient, P (33). The partition coefficient is the ratio of the proportion of the Tf receptor on the cell surface ([TfR]PM) relative to the proportion inside the cell ([TfR]in). In a simple two-compartment model of endocytic trafficking, the partition coefficient equals the ratio of the rate constants for exocytosis and endocytosis (P = [TfR]PM/[TfR]in = kex/ken). The measured partition coefficients were Pbasal = 0.2 and Pinulin = 0.34; thus the calculated endocytic rate constants for the Tf receptor were kex basal = 0.6 ± 0.05 min⁻¹ and ken insulin = 0.59 ± 0.04 min⁻¹, again in good agreement with previously published values (33, 34).

To calculate ken for Glut4, we developed a reversible probe for measuring the partition coefficient of Glut4 in adipocytes (Fig. 3). In these experiments, disulfide-linked Alexa647 was conjugated to anti-HA antibody. Reversible labeling was con-

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firmed by efficient removal of all signals by glutathione treatment when cells were labeled at 4 °C to prevent internalization (Fig. 3B). To measure the partition coefficient, adipocytes were labeled continuously at 37 °C for 4 h and then placed on ice. Half of the samples were treated with glutathione to cleave surface label. Untreated/uncleaved cells show the total amount of labeled Glut4 ([Glut4]total), although cells treated with glutathione give the proportion of Glut4 inside the cells ([Glut4]in). Subtracting [Glut4]in from [Glut4]total gives [Glut4]PM. In insulin-stimulated cells P = [Glut4]PM/[Glut4]in = 0.24/0.76 = 0.32. Thus, the endocytic rate constant for Glut4 in insulin-stimulated cells was kex = 0.04/0.32 = 0.125 min⁻¹. Thus, Glut4 is internalized five times slower than the Tf receptor in insulin-stimulated cells, indicating that these proteins are processed differently at the plasma membrane (34).

It was not possible to measure the partition coefficient in basal cells using this method. The amount of Glut4 in the plasma membrane was too small of a fraction of the total label to accurately measure as a loss of signal after glutathione treatment. However, the partition coefficient could be calculated based on the relative amounts of Glut4 in the plasma membranes of basal and insulin-stimulated cells, which could be accurately measured (Fig. 3B). In these experiments, the amount of Glut4 on the cell surface in basal cells was 12 times less than in insulin-stimulated cells (surface 4 °C). Thus, only 2% of the total Glut4 is found in the plasma membrane in basal cells, compared with 24% in the insulin-stimulated cells. Because the amount of cycling Glut4 in basal cells is one-third that in insulin-stimulated cells (Ymax = 0.33; Fig. 1A), the amount of Glut4 in the plasma membrane is 6% of the total cycling Glut4 ([Glut4]PM = 0.06), and the partition coefficient, P = 0.06/0.94 = 0.064. This gives a calculated value for the endocytic rate constant kex = 0.008/0.064 = 0.125 min⁻¹. These data indicate that insulin does not significantly affect the rate of internalization of Glut4.

As an alternative method to measure the kex for Glut4, we used transition assays. In these assays, Glut4 trafficking was perturbed in cells at steady state, and the kinetics of relaxation to the new steady state were measured (29–31, 41). Two transitions were measured as follows: the basal to insulin transition, and the insulin + inhibitor transition (Fig. 4). In the basal to insulin transition experiments, insulin was added to basal cells at t = 0, and the levels of Glut4 in the plasma membrane were measured at the indicated times. In these experiments, the observed relaxation rate constant, kobs = kex + k−ex = 0.16 + 0.014 min⁻¹. Thus, kex = 0.16 − 0.04 = 0.12 min⁻¹. In the insulin + inhibitor transition experiments, the PI3K inhibitor LY294002 was added to insulin-stimulated cells at t = 0, and the levels of Glut4 in the plasma membrane were measured at the indicated times. The inhibitor decreases kex 10-fold, with little effect on k−ex (Fig. 1A) (30, 31). Therefore, addition of this inhibitor causes a rapid loss of surface Glut4. In the insulin + inhibitor transition experiments, kobs also equals k−ex but kex is very small (~0.004 min⁻¹), so kobs = k−ex = 0.13 ± 0.015 min⁻¹.

To accurately calculate rate constants from partition coefficients, it is essential to eliminate any of the noncycling Glut4 (Glut4 stored in GSVs) from the total and from the intracellular pool.
marker, we compared the endocytic rate constants of LRP-1 and the Tf receptor. The endocytic rate constant for LRP-1 can be determined from $\alpha$-2-M uptake assays. In these experiments, cells are incubated in the continuous presence of Alexa647-labeled $\alpha$-2-M, and the accumulation of $\alpha$-2-M was determined at increasing times (Fig. 6). The kinetics of labeling of cells with $\alpha$-2-M are different from those observed for labeling with Alexa647-Tf or anti-HA antibody (Glut4). Unlike Tf or anti-HA, $\alpha$-2-M dissociates from its receptor in the low pH environment of the endosome. Although the receptor is recycled to the cell surface, the $\alpha$-2-M is retained within the cell and is eventually delivered to the lysosomes for degradation. Thus, $\alpha$-2-M uptake is linear, with continuous uptake occurring with each cycle of the receptor (Fig. 6A). This is in contrast to the uptake of Tf or anti-HA, which is exponential, and saturates after the receptors undergo a single round of trafficking (Figs. 1A and 2A). The rate of $\alpha$-2-M uptake is faster in insulin-stimulated adipocytes than in basal adipocytes. However, there are 40% more $\alpha$-2-M receptors on the cell surface in insulin-stimulated cells than in basal cells. If the data are corrected for the number of receptors on the surface, then the slope of the line ln/Sur versus time plot equals $k_{en}$ (Fig. 6B). The measured endocytic rate constants for $\alpha$-2-M were $k_{en_{basal}} = 0.09 \pm 0.004 \text{ min}^{-1}$ and $k_{en_{insulin}} = 0.12 \pm 0.005 \text{ min}^{-1}$. Thus, the internalization of the $\alpha$-2-M receptor was 5–7 times slower than the Tf receptor in adipocytes, although the internalization rates of $\alpha$-2-M receptor and Glut4 were similar. However, insulin increased the rate of endocytosis of the $\alpha$-2-M receptor, in contrast to what was observed for Glut4. These data are in good agreement with previously published data (42, 43).

To measure the partition coefficients of LRP-1, cells were treated with chloroquine (60 $\mu$M) during the incubation with Alexa647-$\alpha$-2-M, and labeling was continued until saturation (Fig. 6C). Chloroquine neutralizes the low pH of the acidified endosomes, blocking dissociation of $\alpha$-2-M from its receptor (36, 37). In these experiments, insulin caused a 2-fold increase

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in the total amount of α-2-M taken up by the cells (intracellular labeling). Insulin also increased the amount of α-2-M bound to the plasma membrane 1.5-fold. Overall, there was a 1.7-fold increase in the total amount of receptor that was labeled after insulin stimulation, indicating that there was 1.7 times more receptor cycling after insulin stimulation than in basal cells. This increase in the cycling pool size is consistent with the observation that LRP-1 co-localizes to a large extent with Glut4 in the noncycling GSVs (15). The measured partition coefficients ($P = [\text{α-2-M}]_{\text{PSW}}/[\text{α-2-M}]_{\text{in}}$) were $P_{\text{basal}} = 0.5 \pm 0.15$ and $P_{\text{insulin}} = 0.41 \pm 0.05$. The calculated exocytic rate constants were $k_{\text{ex,basal}} = 0.041 \pm 0.008 \text{ min}^{-1}$ and $k_{\text{ex,insulin}} = 0.046 \pm 0.001 \text{ min}^{-1}$. Thus, although Glut4 and LRP-1 are highly co-localized in cells, there are differences in their trafficking.

**DISCUSSION**

It is critically important to understand the extent to which Glut4 is trafficked in the general endocytic pathway versus in specialized pathways. Any protein or pathway that affects the level of Glut4 in the plasma membrane is a potential therapeutic target for the treatment of diabetes. However, it is highly desirable to target Glut4 specifically, without affecting other proteins that are trafficking in the general pathways. It is clear that in adipocytes Glut4 traffics through the general endocytic and secretory pathways (4–8). However, there must be specializations that allow for basal intracellular retention and insulin-stimulated release of Glut4. Exactly where these pathways overlap and to what extent remain unclear. One key marker protein is the Tf receptor, which follows the early/sorting/recycling endosomal pathway. There is clearly some overlap between Glut4 and the Tf receptor based on co-immunoprecipitation and ablation experiments (13, 14). However, these markers are largely distinct in cells. For example, there is very little co-localization seen between Glut4 and the Rab5- and EE1A-positive early endosomes, although there is a high degree of co-localization of Tf with these markers (44–46). Both Glut4 and the Tf receptor are found in the Rab11-positive recycling endosomes (13), which accounts for some of the identified overlap. The Tf receptor is largely excluded from the specialized GSV storage compartments. In contrast, Glut4 is highly co-localized with the late endosomal marker LRP-1 (the α-2-M receptor expressed in adipocytes), in both the general endosomal pathway and in the specialized GSVs. Glut4 also co-localizes with other markers of the late endosomes (the cation-independent mannose 6-phosphate/IGF-II receptor and Rab7) (16, 18, 46). However, although it is relatively easy to show co-localization, it is not easy to determine the extent to which the proteins are following the same or different trafficking itineraries in cells. In this study, we compared the rates of trafficking of Glut4, the Tf receptor, and the α-2-M receptor/LRP-1 to explore the extent to which Glut4 trafficking functionally overlaps with the mechanisms utilized by these other marker proteins.

Proteins destined for the late endosomes such as LRP-1 are sorted from early endosomal proteins like the Tf receptor. This separation begins at the plasma membrane. Receptors that deliver material to the late endosomes for degradation in the lysosomes (such as the LDL, EGF, and α-2-M receptors) are internalized utilizing different adaptor proteins than the Tf receptor (21–25). In some cell types, the different adaptors and their bound cargo are sorted into different clathrin-coated pits (24). Although all clathrin-coated pits contain the Tf receptor, a subset (15–20% of the total) is enriched in the LDL and EGF receptors. After internalization, the vesicles derived from these two classes of clathrin-coated pits are largely kept separated. The majority of the Tf (85%) is delivered to large, relatively immobile Rab5- and EE1A-positive early endosomes, although the LDL and EGF (and 15% of the Tf) are delivered to a distinct population of smaller, rapidly moving early endosomes (Rab5- and Rab7-positive) that mature into late endosomes (Rab7-positive). This segregation requires microtubules; the early and late endosomal proteins were completely co-localized after treatment with nocodazole. Receptors (including the Tf receptor) are recycled from this late endosomal compartment, whereas the ligands are delivered to the lysosomes for degradation. The receptors traffic back to the plasma membrane through the Rab11-positive sorting endosomes, where they are co-localized with markers from the rapidly cycling pathway. Although this degree of separation is not observed in all cell types, our data are consistent with there being two functionally distinct pathways for clathrin-mediated endocytosis in adipocytes, one that is specialized for very fast cycling (the early endosomal pathway followed by the Tf receptor) and a slower cycling pathway that delivers materials to late endosomes/lysosomes and the GSVs (α-2-M/LRP-1 and Glut4).

Knockdown of the clathrin adaptor protein AP2 completely inhibited internalization of the Tf receptor and inhibited the formation of the Tf receptor-specific clathrin-coated pits. However, AP2 knockdown did not affect the uptake of LDL or EGF (these proteins utilize alternative adaptor proteins for internalization) (21, 22, 26). Interestingly, AP2 knockdown in adipocytes significantly inhibited endocytosis of the Tf receptor but had much less of an effect on Glut4 (26). Although this was interpreted to mean that Glut4 is internalized through a mechanism that does not require clathrin, Glut4 is clearly observed in clathrin-coated structures (47–50). The role of clathrin in Glut4 endocytosis is difficult to study directly (51), because clathrin is essential for the proper intracellular sorting of Glut4 (52). Knockdown of the clathrin heavy chain significantly slowed the trafficking of Glut4 through intracellular compartments, causing a redistribution of Glut4 from the plasma membrane to intracellular compartments. In contrast, although clathrin knockdown inhibited Tf internalization, it had little effect on the intracellular trafficking of the TfR. This would clearly complicate interpretation of the internalization of Glut4 (amount accumulated inside of the cells) in clathrin heavy chain knockdown experiments. Thus, it is possible that like the late endosome markers, Glut4 is internalized through a distinct subset of clathrin-coated pits, utilizing an alternative set of adaptor proteins than AP2. If this were the case, then Glut4 would still be internalized/co-localized with the Tf receptor (consistent with the ablation studies), but it would be co-localized with only the small fraction of the Tf receptor that is internalized through the late endosomal pathway.

Consistent with the idea that Glut4 and Tf are endocytosed through different pathways, the kinetics of internalization of Tf and Glut4 are very different. The endocytic rate constant for Tf
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A series of differential equations describing a two-compartment model for antibody binding and uptake were solved by numerical methods using the program Mathematica as described previously (3). Simulated data for anti-HA uptake as well as intracellular (In) and plasma membrane-bound (Sur) labeled antibody were generated from 0 to 600 min under basal (■) or insulin-stimulated (□) conditions. Values used for the rate constants are as follows: $k_{ex\ basal} = 0.003 \text{ min}^{-1}; k_{ex\ insulin} = 0.08 \text{ min}^{-1}$ (12); and $k_{en\ basal} = k_{en\ insulin} = 0.12 \text{ min}^{-1}$ (Fig. 4). A, uptake experiment, line, single exponential fits were used to calculate $k_{obs\ basal} = 0.0028 \text{ min}^{-1}, k_{obs\ insulin} = 0.078 \text{ min}^{-1}$. B, endocytosis, In/Sur, early time points. Line, single exponential fits; slopes, basal = 0.21, insulin = 0.085. C, endocytosis, In/Sur, full time course. Line, single exponential fits; $k_{obs\ basal} = 0.0028 \text{ min}^{-1}, k_{obs\ insulin} = 0.078 \text{ min}^{-1}$. D, Tf endocytosis assay, simulated data for Tf binding at 4°C, was $\approx 0.6 \text{ min}^{-1}$ in both basal and insulin-stimulated cells ($t_{1/2} = 1–2 \text{ min}$) (33). From our kinetics data, we estimate Glut4 $k_{en}$ $\approx 0.12 \text{ min}^{-1}$ in both basal and insulin-stimulated cells ($t_{1/2} = 5–6 \text{ min}$). This endocytic rate constant for Glut4 was determined using multiple independent methods (Figs. 3–5). Therefore, Tf is internalized 5 times faster than Glut4 in adipocytes, strongly supporting the hypothesis that Glut4 and Tf are internalized via different mechanisms. Consistent with this, Glut4 was also internalized $\approx 5$ times slower than the Tf receptor in cardiomyocytes, indicating differences in the processing of these two proteins at the level of clathrin vesicle formation in muscle cells as well (34). The late endosomal marker LRP-1 ($\alpha$-2-M receptor) was also internalized at a slower rate than the Tf receptor in adipocytes ($k_{en\ basal} = 0.09 \text{ min}^{-1}, k_{en\ insulin} = 0.12 \text{ min}^{-1}$; Fig. 6). This suggests that Glut4 and LRP-1 may be internalized and trafficked together, consistent with the high degree of co-localization seen with these two proteins (15).

One of the controversial questions remaining in Glut4 trafficking is whether insulin regulates the endocytosis of Glut4. We see no difference in $k_{en}$ in basal or insulin-stimulated cells using a number of independent methods to measure or calculate the rate constant for internalization of Glut4. This is in disagreement with previous studies, which reported that insulin decreases the endocytic rate constant of Glut4 in adipocytes 2–3-fold (26, 53). However, this conclusion was based on the inappropriate application of the mathematical analysis of Wiley and Cunningham (32) to calculate relative $k_{en}$ values from internalization data. For proteins that dissociate from their receptors after internalization, such as EGF or LDL or $\alpha$-2-M, the accumulation of ligand with time is linear after the receptors reach equilibrium and before loss of signal due to degradation in the lysosomes (Fig. 6A) (32). To calculate endocytic rate constants, the rate of internalization is corrected for the total number of cell surface receptors (Fig. 6B). The slope of internal/surface versus time plot equals $k_{en}$. However, for ligands that do not dissociate from their receptors upon internalization, such as anti-HA antibody bound to epitope-tagged Glut4 or Tf bound to the Tf receptor, uptake is exponential, reaching a maximum when the ligand binding to the receptors reaches equilibrium; this maximum depends on the concentration of ligand, the “on” and “off” rates of ligand binding, as well as the rates of exocytosis and endocytosis of the receptor. The simple analysis of the slope of the In/Sur plot cannot be applied to internalization of these types of ligands. To illustrate this, we simulated the data that would be observed in the assays described in the previous papers, using their reported exocytic rate constants ($k_{ex\ basal} = 0.003 \text{ min}^{-1}, k_{ex\ insulin} = 0.08 \text{ min}^{-1}$) (12) and our measured endocytic rate constant ($k_{en} = 0.12 \text{ min}^{-1}$) (Fig. 7). This simulation accurately recreates the reported data for both an uptake experiment (Fig. 7A) and an internalization experiment (Fig. 7B).

The simulated data generated for the internalization assay is reasonably well fit by a line over the time course used in the previous studies (the “early” time points; basal, 1–30 min; insu-
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lin, 1–8 min). As was reported, insulin causes a 2.5-fold decrease in the slope of the In/Sur plot in the simulated data. However, the decrease in the slope in these simulations is due entirely to an increase in $k_{en}/k_{ex}$ was kept constant. When the internalization simulation is extended for longer times (basal, 1–300 min; insulin, 1–100 min), it is clear the data curve is exponential and not linear (Fig. 7C). The relaxation of the system from the initial state to the fully labeled state is dependent on the exocytosis of Glut4, at all times; the In/Sur plot is an inverse function of the partition coefficient ($1/P = k_{en}/k_{ex}$). Although changes in $k_{en}$ would cause a change in the internalization assay described, the relationship between the slope of the linear fit of the early time points and the magnitude of change in $k_{en}$ is complex and is not directly proportional. Furthermore, changes in $k_{ex}$ also affect the slope of the line. Therefore, it remains unclear whether there was actually a decrease in $k_{en}$ observed in the previously published experiments. The In/Sur method has also been inappropriately applied to the analysis of Tf endocytosis in many publications. The interpretation of the results of “internalization assays” using labeled Tf to monitor trafficking of the Tf receptor is even more difficult, because Tf dissociates from its receptor when it returns to the cell surface, which makes the data very complicated (Fig. 7D). Clearly Tf uptake is nonlinear; therefore, a linear fit of the data is inappropriate over any time course. To avoid this problem, we and others have used the partition coefficient to calculate $k_{en}$ using measured values of $k_{ex}$ (33).

In addition to differences in the rates of internalization, the rates of cycling of Glut4 and the Tf receptor are also very different. Under identical experimental conditions, the measured exocytic rate constant for Glut4 in basal cells ($k_{ex} = 0.008 \text{ min}^{-1}$; Fig. 1A) was 15 times slower than the exocytic rate constant for the Tf receptor ($k_{ex} = 0.12 \text{ min}^{-1}$; Fig. 2B). Glut4 cycling was still five times slower than the Tf receptor after insulin stimulation (Glut4 $k_{ex} = 0.04 \text{ min}^{-1}$; Tf receptor $k_{ex} = 0.20 \text{ min}^{-1}$). The differences in the rates of endocytosis and exocytosis indicate that the cycling Glut4 and the Tf receptor are processed differently at both the cell surface and in intracellular pathways, despite some clear overlap in the protein machinery and compartments used in the trafficking of both proteins. This means that either the majority of the Glut4 does not traffic together with the majority of the Tf receptor through the early endosomal pathway or that there are specialized mechanisms that retard the trafficking of the Glut4.

The kinetics data as well as co-localization studies suggest the model depicted in Fig. 8. The data suggest that most of the Tf/Tf receptor (TfR) is internalized through a rapid mechanism that is distinct from the one utilized by Glut4 and LRP-1/$\alpha$-2-M. The TfR remains largely segregated from these other markers and is rapidly recycled back to the plasma membrane after release of iron in the mildly acidic endosomes. A small fraction of the TfR cycles through the slower late endosomal/GSV cycling pathway, accounting for the observed co-localization with Glut4 (ablation and co-immunoprecipitation studies) and the slow cycling pool (Fig. 2A). In contrast, both co-localization studies and the kinetics data suggest that Glut4 and LRP-1 are internalized together utilizing the same mechanism. However, in basal cells LRP-1 is rapidly recycled to the plasma membrane after release of $\alpha$-2-M in the more acidic late endosomes ($t_{1/2} = 17 \text{ min}$), although the small pool of cycling Glut4 is recycled much more slowly ($t_{1/2} = 90 \text{ min}$). Therefore, Glut4 must be sorted from the majority of the LRP-1 into compartments that cycle slowly. Both LRP-1 (40–50%) and Glut4 (80–90%) are sequestered in the static GSVs in basal cells. Insulin mobilizes the Glut4 and LRP-1 in the GSVs and inhibits the sorting of Glut4 away from LRP-1 in the endosomes. In insulin-stimulated cells, Glut4 and LRP-1 may recycle back to the PM together ($t_{1/2} = 15 \text{ min}$), but they remain segregated from the majority of recycling TfR ($t_{1/2} = 3 \text{ min}$).

![Model](https://example.com/model.png)

**FIGURE 8. Model.** In basal cells, the cycling Glut4 (10–20%) and LRP-1 (50–60%) are internalized via the same mechanism ($t_{1/2} = 6–7 \text{ min}$), although the majority of the Tf/Tf receptor (TfR) is internalized via a different, faster pathway ($t_{1/2} = 1–2 \text{ min}$). Iron is released from the Tf in the mildly acidic early endosome, and the Tf is rapidly recycled back to the plasma membrane (PM) with its receptor ($t_{1/2} = 5–6 \text{ min}$). After internalization, $\alpha$-2-M dissociates from LRP-1 in the more acidic late endosomes and is delivered to the lysosomes for degradation, although the LRP-1 is recycled back to the cell surface ($t_{1/2} = 15–20 \text{ min}$). Although co-localized initially, Glut4 is sorted from the LRP-1 in the endosomes and is returned to the cell surface via a slow insulin-sensitive pathway ($t_{1/2} = 90 \text{ min}$). Both Glut4 (80–90%) and LRP-1 (40–50%) are sequestered in the noncycling static GSVs in basal cells. Insulin mobilizes the Glut4 and LRP-1 in the GSVs and inhibits the sorting of Glut4 away from LRP-1 in the endosomes. In insulin-stimulated cells, Glut4 and LRP-1 may recycle back to the PM together ($t_{1/2} = 15 \text{ min}$), but they remain segregated from the majority of recycling TfR ($t_{1/2} = 3 \text{ min}$).
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receptor is co-localized with Glut4 in the slower cycling endosomal pathway. Thus, it should be possible to increase the amount of Glut4 in the plasma membrane without affecting the trafficking of Tf and its receptor. The degree of functional overlap between the late endosomal pathway and the cycling Glut4, as well as the overlap between the static GSVs, and this pathway remains to be determined.

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