GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin

Vincent Blot and Timothy E McGraw*

Department of Biochemistry, Weill Medical College of Cornell University, New York, NY, USA

Insulin slows GLUT4 internalization by an unknown mechanism. Here we show that in unstimulated adipocytes, GLUT4 is internalized by two mechanisms. Approximately 80% of GLUT4 is internalized by a mechanism that is sensitive to the cholesterol-aggregating drug nystatin, and is independent of AP-2 clathrin adaptor and two putative GLUT4 endocytic motifs. The remaining GLUT4 is internalized by an AP-2-dependent, nystatin-resistant pathway that requires the FQI GLUT4 motif. Insulin inhibits GLUT4 uptake by the nystatin-sensitive pathway and, consequently, GLUT4 is internalized by the AP-2-dependent pathway in stimulated adipocytes. The phenylalanine-based FQI GLUT4 motif promotes AP-2-dependent internalization less rapidly than a tyrosine-based motif, the classic form of aromatic-based motifs. Thus, both a change in the predominant endocytosis pathway and the specific use of a suboptimal internalization motif contribute to the slowing of GLUT4 internalization in insulin-stimulated adipocytes. Insulin also inhibits the uptake of cholera-toxin B, indicating that insulin broadly regulates cholesterol-dependent uptake mechanisms rather than specially targeting GLUT4. Our work thus identifies cholesterol-dependent uptake as a novel target of insulin action in adipocytes.

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Introduction

Insulin regulates glucose transport in adipose and muscle cells by modulating the amount of the GLUT4 glucose transporter in the plasma membrane (Watson *et al*, 2004; Dugani and Klip, 2005). In unstimulated basal adipocytes, less than 5% of GLUT4 is in the plasma membrane. A dynamic process involving slow GLUT4 exocytosis and fast GLUT4 internalization determines the steady-state distribution of GLUT4 between the plasma membrane and intracellular compartments. Insulin acts by altering the rates of GLUT4 trafficking between intracellular compartments and

the plasma membrane, resulting in a net increased accumulation of GLUT4 in the plasma membrane. At the new steady state in the presence of insulin, about 50% of GLUT4 is in the plasma membrane. The effects of insulin on GLUT4 exocytosis have been extensively studied and well documented (e.g., Govers *et al*, 2004; Karylowski *et al*, 2004; Martin *et al*, 2006).

The GLUT4 internalization mechanism and the GLUT4 sequences that determine internalization have not been fully described, nor is it known how insulin inhibits GLUT4 endocytosis. There are data documenting a role for clathrin-coated pits, as well as data supporting a role for cholesterol-enriched domains in GLUT4 endocytosis (e.g., Robinson *et al*, 1992; Ros-Baro *et al*, 2001; Shigematsu *et al*, 2003). Two motifs have been proposed to mediate GLUT4 endocytosis: an F⁵QI sequence in the GLUT4 amino-terminal domain (Piper *et al*, 1993; Garippa *et al*, 1994; Araki *et al*, 1996; Al-Hasani *et al*, 2002; Govers *et al*, 2004) and an LL⁴⁹⁰ sequence in the carboxyl-terminal domain (Czech and Buxton, 1993; Verhey *et al*, 1995; Garippa *et al*, 1996; Govers *et al*, 2004). The F⁵QI motif is a member of the aromatic-based internalization motif family and the LL⁴⁹⁰ sequence is a member of the LL-based family of trafficking motifs (Bonifacino and Traub, 2003). Both these classes of motifs have been implicated in regulating internalization from the plasma membrane as well as targeted intracellular trafficking (Bonifacino and Traub, 2003).

In this study, we have further characterized GLUT4 endocytosis in adipocytes. We found that GLUT4 is internalized in basal adipocytes by two mechanisms. The main pathway, accounting for about 80% of basal endocytosis, is sensitive to the cholesterol-aggregating drug nystatin and independent of the AP-2 clathrin adaptor and the F⁵QI and LL⁴⁹⁰ GLUT4 endocytic motifs. The second GLUT4 internalization pathway is nystatin-resistant and dependent on AP-2 and the F⁵QI motif. Both endocytic mechanisms contribute to GLUT4 internalization in basal conditions. Insulin inhibits the nystatin-sensitive pathway and, in stimulated cells, GLUT4 is only internalized by the nystatin-resistant, AP-2-dependent pathway. The F⁵QI endocytosis motif functions in a suboptimal manner compared to more conventional tyrosine-based motifs and, therefore, GLUT4 uptake by the AP-2 pathway is slow. Thus, both a change in the predominant mechanism for uptake and the specific use of a suboptimal internalization motif contribute to the slowing of GLUT4 internalization in insulin-stimulated adipocytes.

Results

Insulin inhibits GLUT4 internalization

We used HA-GLUT4-GFP as surrogate for GLUT4 trafficking. This construct contains an HA epitope in the first exofacial loop and a GFP fused to the carboxyl cytoplasmic domain (Figure 1A; Lampson *et al*, 2000). We measured basal and

*Corresponding author. Department of Biochemistry, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA. Tel.: +1 212 747 4982; Fax: +1 212 747 8875; E-mail: temcgrow@med.cornell.edu

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Figure 1 Insulin inhibits GLUT4 endocytosis. (A) Schematic representation of the HA-GLUT4-GFP reporter. The F⁵QQI and LL⁴⁹⁰ motifs are noted. The positions of the amino acids from the amino terminus are shown. (B) Representative experiment determining GLUT4 internalization rate constant in adipocytes. Internalization of HA-GLUT4-GFP in insulin-stimulated or basal adipocytes was measured by determining the ratio of HA.11 anti-HA antibody internalized normalized to the amount of HA.11 on the plasma membrane. The slope of the plot of the internal-to-plasma membrane ratio versus time is the internalization rate constant. The values are the mean $\pm$ s.e.m. determined from at least 20 cells per time point. Different time courses for measurement of GLUT4 internalization in the basal and insulin-stimulated conditions are discussed as Supplementary data. (C) Insulin inhibits GLUT4 endocytosis. Results are the mean $\pm$ s.d. deviation of 10 independent experiments measuring GLUT4 internalization in basal and insulin-stimulated conditions. The data were normalized to the basal rate of each experiment. (D) Elevated level of GLUT4 in the plasma membrane of insulin-stimulated adipocytes does not saturate GLUT4 uptake mechanism. Results are cell-by-cell analysis from the 9 min time point of five independent GLUT4 internalization assays in insulin-stimulated adipocytes. Within each experiment, surface GLUT4 and the corresponding internalized GLUT4 are normalized to the average values.

insulin HA-GLUT4-GFP internalization by quantifying HA.11 monoclonal anti-HA antibody uptake using the internal/surface (IN/SUR) method (Wiley and Cunningham, 1982). This method requires that the pulse times in HA.11 be chosen to minimize the recycling of internalized HA.11 to the plasma membrane during the pulse periods (see Materials and methods). In insulin-stimulated cells, HA-GLUT4-GFP recycles with a half-time of about 10–15 min, and internalization pulse times of less than 15 min were used. In basal cells, the GLUT4 recycling half-time is on the order of 100 min and therefore longer pulse times can be used. Because the amount of HA-GLUT4-GFP in the plasma membrane of basal adipo-

Table 1 Basal and insulin endocytosis rate constants of wild-type and mutated GLUT4

GLUT4	Basal internalization rate constant (min ⁻¹)	Insulin internalization rate constant (min ⁻¹)
HA-GLUT4 GFP WT	0.20 $\pm$ 0.002	0.06 $\pm$ 0.007
HA-GLUT4 GFP F ⁵ A	0.17	0.03 $\pm$ 0.003
HA-GLUT4-GFP F ⁵ Y	0.20 $\pm$ 0.006	0.29 $\pm$ 0.017
HA-GLUT4-GFP L ⁴⁹⁰ A	0.19	0.05

cytes is low (about a tenth of that in insulin-stimulated cells), the amount of HA.11 internalized per unit time is also low. Thus, pulse times up to 30 min were used to measure basal internalization.

The amounts of internal and plasma membrane HA.11 were measured using quantitative fluorescence microscopy (Lampson *et al*, 2001; Zeigerer *et al*, 2002, 2004; Martin *et al*, 2006), and HA-GLUT4-GFP internalization rate constants were derived from a plot of internal α HA.11/surface HA.11 versus time (Figure 1B). Because the amount of α HA.11 uptake is normalized to the level of plasma membrane HA.11, the IN/SUR method measures internalization independent of exocytosis (Materials and methods; Wiley and Cunningham, 1982). GLUT4 was internalized in the basal state with a rate constant of $0.20 \pm 0.002 \text{ min}^{-1}$ and internalization was reduced to $0.06 \pm 0.007 \text{ min}^{-1}$ in the presence of insulin (Figure 1B and Table 1). The average effect of insulin was to inhibit GLUT4 endocytosis by $60 \pm 8\%$ (Figure 1C). Moreover, basal IN/SUR measurements performed on an overlapping timescale with that used for insulin fit the same line as basal IN/SUR measurements made at longer pulse times (Supplementary Figure 1). These data indicate that small differences in the time course for uptake in basal and insulin conditions did not account for the differences in GLUT4 internalization in the two conditions.

Because insulin treatment results in an ~ 10 -fold increase in GLUT4 in the plasma membrane, the slower internalization rate of GLUT4 in the insulin-stimulated state could possibly result from saturation of the endocytic machinery. To rule out this possibility, we plotted internal HA.11 versus surface HA.11 of individual cells (Figure 1D). The linear correlation observed between the amount of GLUT4 on the cell surface and the amount of GLUT4 internalized over an ~ 10 -fold range of surface expression indicates that the increased GLUT4 on the surface of insulin-stimulated adipocytes does not saturate the uptake mechanism.

GLUT4 mutagenesis reveals two distinct endocytosis pathways in the basal and insulin-stimulated states

To further explore the molecular basis of GLUT4 internalization, we characterized the behaviors of HA-GLUT4-GFP constructs in which the two candidate GLUT4 internalization motifs have been mutated: the amino-terminal F⁵QQI motif (F⁵A mutant), the carboxyl-terminal LL⁴⁹⁰ motif (L⁴⁹⁰A mutant) or both motifs (F⁵A/ L⁴⁹⁰A) (Figure 1A). The ranges of expressions and the patterns of intracellular localization of the mutants were indistinguishable from those of wild-type HA-GLUT4-GFP, with all constructs concentrating in the perinuclear region of the cells (Figure 2A). The F⁵A mutation had a small inhibitory effect on basal GLUT4 endocytosis ($13 \pm 7\%$ inhibition), whereas the L⁴⁹⁰A mutation had

Figure 2 GLUT4 follows two different internalization pathways in the basal and insulin-stimulated state. (A) GLUT4 mutant localizations. Localization of different HA-GLUT4-GFP constructs expressed in adipocytes and detected by GFP fluorescence in epifluorescence microscopy. (B, C). GLUT4 internalization in basal adipocytes (B) and insulin-stimulated adipocytes (C). The results are the mean $\pm$ s.d. of at least three independent experiments. The data were normalized to the basal rate of wild-type HA-GLUT4-GFP measured in each experiment. * $P < 0.05$, ** $P < 0.01$; Student's *t*-test probabilities.

no impact (Figure 2B, and Supplementary Figure 2). The F^{5A}/L^{490A} double mutation did not affect basal endocytosis more than the single F^{5A} mutation, ruling out the possibility that the motifs are functionally redundant. In insulin-stimulated adipocytes, the F^{5A} mutant was internalized at about 50% of wild-type HA-GLUT4-GFP, whereas the L^{490A} mutant was internalized at the same rate as wild type (Figure 2C). The F^{5A}/L^{490A} double mutant was internalized at the same slow rate as the F^{5A} mutant. The internalization rate constant of the F^{5A} mutant in the insulin-stimulated state was $0.03 \pm 0.003 \text{ min}^{-1}$ (Table I), which is comparable to bulk membrane uptake measured in other cell types (Mayor *et al.*, 1993). These data indicate that the F⁵QQI motif has a minor role in basal GLUT4 internalization and becomes essential in insulin-stimulated adipocytes, and thereby suggest that GLUT4 is internalized by distinct mechanisms in basal and insulin-stimulated adipocytes.

GLUT4 endocytosis is AP-2-independent in resting adipocytes and becomes AP-2-dependent upon insulin stimulation

The F⁵QQI motif, a member of the aromatic-based internalization motif family (Bonifacino and Traub, 2003), has been shown to bind the $\mu 2$ chain of AP-2 clathrin adaptors in yeast two-hybrid experiments (Al-Hasani *et al.*, 2002). Because the data in Figure 2 demonstrated a major role for the F⁵QQI motif in GLUT4 endocytosis solely after insulin stimulation, we hypothesized that the difference between GLUT4 internalization mechanisms in basal and insulin-stimulated states may be due to different requirements for AP-2.

To specially assess the role of AP-2 in GLUT4 internalization, we knocked down AP-2 in adipocytes using a small hairpin RNA targeting the $\mu 2$ subunit of the AP-2 complex (pSUPER- $\mu 2$; Dugast *et al.*, 2005). Knock down of $\mu 2$ results in a concomitant reduction of the whole AP-2 complex (Motley *et al.*, 2003; Huang *et al.*, 2004). We monitored the AP-2 knockdown efficiency in transfected adipocytes by quantifying AP-2 α chain in indirect immunofluorescence (Figure 3A). In control cells, there was a broad range of expression of AP-2 among individual cells (Figure 3B). In adipocytes transfected with pSUPER- $\mu 2$, the average AP-2 immunofluorescence per cell was about one-third of the average value in control cells (Figure 3B), demonstrating an efficient knockdown of AP-2. The AP-2 knockdown resulted in a two-fold reduction in transferrin receptor (TR) internalization (Figure 3C). This measurement is likely to be an underestimate because the AP-2 knockdown was incomplete and, indeed, the amount of AP-2 remaining in the knockdown cells correlated with the amount of internalized transferrin (Figure 3D). Thus, although incomplete, the level of $\mu 2$ knockdown we achieved in differentiated adipocytes is sufficient to induce a perturbation of AP-2-dependent internalization.

AP-2 knockdown resulted in an $\sim 50\%$ inhibition of GLUT4 endocytosis in insulin-stimulated adipocytes but had no effect on basal adipocytes (Figure 4A and B). These data together with those presented in Figure 3 indicate that the FQIQ motif and the AP-2 clathrin adaptor are required for GLUT4 internalization in insulin-stimulated adipocytes, whereas basal GLUT4 internalization is predominantly independent of AP-2 and the FQIQ motif.

GLUT4 in endocytic vesicles colocalize with AP-2 and Tf only after insulin stimulation

In total internal reflection fluorescence (TIRF) microscopy, which reveals GFP fluorescence within 250 nm of the dorsal membrane, HA-GLUT4-GFP was localized to puncta in basal adipocytes (Figure 5A). In insulin-stimulated adipocytes, in addition to GLUT4 concentrated in puncta, there was a significant increase in diffuse GFP fluorescence, as would be the case if GLUT4 was distributed throughout the membrane (Figure 5A). In both basal and insulin-stimulated adipocytes, many of the HA-GLUT4-GFP puncta detected by TIRF microscopy colocalized with HA.11 antibodies internalized for 10 min (Figure 5A, overlay). Those puncta had been derived from the plasma membrane during the 10 min pulse and are therefore of endocytic origin. TIRF microscopy can thus be used to examine colocalization of HA-GLUT4-GFP endocytic vesicles with other endocytic markers.

Endogenous AP-2 localization in HA-GLUT4-GFP-expressing cells was determined by immunofluorescence in epifluorescence mode by choosing a focal plane at the bottom of the cells, and compared to HA-GLUT4-GFP detected in TIRF mode. AP-2 was localized to puncta both in the presence and absence of insulin (Figure 5B). Overlay of TIRF and the epifluorescence images revealed extensive codistribution between HA-GLUT4-GFP puncta and AP-2 puncta in insulin-stimulated adipocytes, and very little overlap in basal conditions (Figure 5B). Unlike wild-type GLUT4, F^{5A} GLUT4 did not colocalize with AP-2 in insulin-stimulated adipocytes (Figure 5B), consistent with the hypothesis that an intact FQIQ motif is required to target GLUT4 to the insulin AP-2-dependent uptake mechanism.

Figure 3 AP-2 clathrin adaptor knockdown in 3T3-L1 adipocytes. (A) AP-2 knockdown in adipocytes. Adipocytes were electroporated with HA-GLUT4-GFP or the human TR plasmid alone (control) or together with the pSUPER μ2 plasmid encoding an shRNA to the AP-2 μ2 chain. Forty-eight hours after electroporation, cells were fixed and stained for AP-2. Images are representative of three independent experiments and have been equally scaled so that pixels intensities can be directly compared. (B) Quantification of AP-2 knockdown. For both control and pSUPER μ2 conditions, AP-2 α chain fluorescence was quantified for greater than 200 cells identified as positive for transfection based on either HA-GLUT4-GFP or human TR expression. Results are pooled data from five independent experiments. Within each experiment, AP-2 α chain fluorescence was normalized to the average value obtained for control cells. (C) Effect of AP-2 knockdown on TR endocytosis. Basal TR internalization rate was measured in control and in pSUPER μ2 electroporated adipocytes. Results are mean with s.d. of two independent experiments. (D) Remaining TR endocytosis is associated with incomplete AP-2 knockdown. Plot of 9 min transferrin uptake versus AP-2 fluorescence in adipocytes electroporated with TR alone (control) or TR and pSUPER μ2. Results are pooled data from three independent experiments. Within each experiment, AP-2 α chain fluorescence was normalized to the average value obtained for control cells. The dashed line shows the correlation between the amount of transferrin uptake and the remaining AP-2 fluorescence in pSUPER μ2 electroporated cells.

To compare HA-GLUT4-GFP puncta to cargo internalized by an AP-2-dependent mechanism, colocalization with transferrin was performed. Vesicles containing HA-GLUT4-GFP showed extensive colocalization with internalized transferrin solely in insulin-stimulated adipocytes, providing additional evidence for the proposal that GLUT4 is internalized by an AP-2-dependent mechanism only in insulin-stimulated conditions (Figure 5C).

GLUT4 is predominantly endocytosed by a cholesterol-dependent, nystatin-sensitive mechanism in basal adipocytes

To further characterize basal GLUT4 endocytosis, we used the cholesterol-sequestering drug nystatin, which is known to inhibit clathrin-independent but not clathrin-dependent internalization (Schnitzer *et al*, 1994). Nystatin did not inhibit TR internalization, confirming that clathrin-dependent endocytosis in adipocytes is not sensitive to nystatin-induced cholesterol aggregation (Figure 6A). Nystatin inhibited basal GLUT4 endocytosis by 80% whereas GLUT4 internalization in insulin-stimulated adipocytes was not affected (Figure 6B). Thus, GLUT4 endocytosis is mainly by a nystatin-sensitive mechanism in basal conditions and by a nystatin-resistant mechanism upon insulin stimulation.

Nystatin did not completely block basal GLUT4 endocytosis, but lowered it to the level of insulin-stimulated adipo-

cytes (Figure 6B). One explanation for these data is that residual GLUT4 internalization in nystatin-treated adipocytes is by the AP-2-dependent pathway. To test this hypothesis, we measured the effect of nystatin on basal endocytosis of F⁵A GLUT4. We found that nystatin reduced basal endocytosis of F⁵A GLUT4 to a level lower than that of wild-type GLUT4 (Figure 6C). These results indicate that, in basal conditions, two GLUT4 internalization mechanisms coexist; the majority (~80%) of GLUT4 is internalized by a nystatin-sensitive mechanism that is independent of the FQI motif, whereas a smaller fraction (~20%) of GLUT4 is endocytosed by nystatin-resistant and FQI motif-dependent endocytosis. After insulin stimulation, GLUT4 internalization is only by the nystatin-resistant, FQI motif-dependent pathway.

Insulin accelerates the nystatin-resistant uptake and inhibits the nystatin-sensitive uptake

The nystatin-sensitive component of basal GLUT4 uptake is inhibited in insulin-stimulated adipocytes (Figure 6B). This could be due either to specific effects of insulin on GLUT4 or to broader effects of insulin on the nystatin-sensitive endocytosis pathway. To distinguish between these possibilities, we determined the effects of insulin on the uptake of cholera-toxin B (CT-B). Insulin, like nystatin, caused a reduction in the amount of CT-B taken up (Figure 7A). CT-B is internalized by both clathrin-independent and clathrin-dependent

Figure 4 GLUT4 internalization is AP-2-independent at basal and becomes AP-2-dependent after insulin stimulation. GLUT4 internalization was measured in control and in AP-2 knockdown cells in both (A) the basal and (B) the insulin-stimulated states. Results are the mean $\pm$ s.d. of three independent experiments.

pathways (Sandvig *et al*, 2004). However, as we have previously reported (Martin *et al*, 2006), insulin did not inhibit clathrin-dependent TR uptake but rather accelerated it (Figure 7B).

Thus, insulin inhibits the nystatin-sensitive endocytosis pathway while accelerating the nystatin-resistant clathrin-dependent uptake. These data support the hypothesis that insulin slows GLUT4 internalization by regulating the nystatin-sensitive uptake pathway rather than specifically slowing GLUT4 internalization.

Slow internalization of GLUT4 upon insulin stimulation is due to the suboptimal nature of the FQOI internalization motif

The FQOI GLUT4 internalization motif is unusual because it contains a phenylalanine instead of a tyrosine (Bonifacino and Traub, 2003). To test if the phenylalanine has a specific role in the characteristics of GLUT4 uptake in insulin-stimulated cells, we examined the behavior of GLUT4 in which a tyrosine was substituted for phenylalanine (F⁵Y mutant). As expected, the F⁵Y mutation did not change basal GLUT4

internalization (Figure 8). However, in insulin-stimulated adipocytes, F⁵Y GLUT4 was internalized more rapidly than wild-type GLUT4, consistent with the phenylalanine-based FQOI motif being a suboptimal motif (Figure 8 and Table I). These data thus indicate that the phenylalanine-5 in GLUT4 is specifically required for the slow internalization of GLUT4 in insulin-stimulated adipocytes.

GLUT4 return-to-basal after insulin withdrawal is a complex process involving the F⁵QOI and LL⁴⁹⁰ motifs as well as nystatin-sensitive pathways

Previous studies have documented a role for the GLUT4 F⁵QOI and LL⁴⁹⁰ motifs in the return of elevated plasma membrane GLUT4 in insulin-stimulated adipocytes to its low basal level following insulin withdrawal or addition of wortmannin (Verhey *et al*, 1995; Govers *et al*, 2004). As the distribution of GLUT4 between the interior and cell surface depends on endocytosis and exocytosis, changes in the rate of return to basal GLUT4 retention will be sensitive to changes in endocytosis, exocytosis or both.

We found that the return to the basal GLUT4 retention was slowed by about 50% for both F⁵A and L⁴⁹⁰A mutants (Figure 9A). The delay we observed for the F⁵A mutant is consistent with the effect of this mutation on GLUT4 endocytosis upon insulin stimulation (Figure 2). By contrast, the L⁴⁹⁰A mutation did not alter GLUT4 internalization in either the basal or the insulin-stimulated states (Figure 2). Thus, the delay in the return to basal retention of this mutant may reflect a role for the LL⁴⁹⁰ motif in an intracellular trafficking step required for establishment of basal retention. If true, the F⁵A and L⁴⁹⁰A mutations would have additive effects on the return to basal retention of GLUT4. Indeed, we found that the return to the basal steady state of the F⁵A/L⁴⁹⁰A double mutated GLUT4 was slower than either of the individual mutants (Figure 9A).

We also examined the effect of nystatin on the return to basal retention of GLUT4. Nystatin greatly reduced the rate of GLUT4 return to basal retention (Figure 9B), documenting a role for the nystatin-sensitive pathway in this process. The effect is consistent with our findings that fast basal GLUT4 internalization is sensitive to nystatin but does not exclude that other intracellular pathways required for the establishment of basal retention are also sensitive to nystatin treatment.

Discussion

We have characterized GLUT4 endocytosis in basal and insulin-stimulated adipocytes. The internalization assay we used here is based on quantification of antibody uptake from the cell surface. Because quantification of internalized antibodies was normalized to the amount of antibody bound to the cell surface on individual cells, this method allows for the analysis of endocytosis independent of any changes in exocytosis (Wiley and Cunningham, 1982). Our data indicate that GLUT4 is internalized by two pathways in basal adipocytes. The majority of GLUT4 is internalized through an AP-2-independent, nystatin-sensitive mechanism that does not involve any known GLUT4 trafficking motifs. A smaller portion of GLUT4 is endocytosed through a nystatin-resistant pathway and requires AP-2 and the GLUT4 F⁵QOI motif (Figure 10A). The larger flux of GLUT4 through the

Figure 5 GLUT4 colocalizes with AP-2 and internalized transferrin in endocytic vesicles only in the insulin-stimulated state. GFP images were collected in the green channel in the total TIRF mode and the Cy-3 channel in the epifluorescence mode (Epi). (A) Colocalization between HA-GLUT4-GFP in the TIRF zone and HA.11 anti-HA internalized for 10 min to label HA-GLUT4-GFP-containing endosomes. (B) Colocalization between HA-GLUT4-GFP in the TIRF zone and AP-2 clathrin adaptor. (C) Colocalization between HA-GLUT4-GFP and Alexa546-conjugated transferrin internalized for 10 min. Overlays of green TIRF images and red epifluorescence images are shown (overlay). Images are representative of results from three independent experiments, and have been scaled by eye, and as a consequence, absolute pixel intensities between different panels cannot be compared.

nystatin-sensitive pathway most likely reflects a higher affinity of GLUT4 for this internalization mechanism. Upon insulin stimulation, GLUT4 uptake is only by the nystatin-resistant, AP-2- and F⁵QQI motif-dependent pathway (Figure 10B). This change in the predominant GLUT4 internalization pathway results in a three-fold decrease of GLUT4 endocytosis.

We found that insulin also slows the uptake of CT-B, which is endocytosed by both clathrin-dependent (nystatin-resistant) and clathrin-independent (nystatin-sensitive) pathways (Sandvig *et al*, 2004). Because insulin does not inhibit the clathrin-dependent internalization (Figure 7A), we conclude

that insulin selectively inhibits the nystatin-sensitive uptake pathway. We thus propose that when the nystatin-sensitive pathway is inhibited by insulin, AP-2-dependent nystatin-resistant endocytosis becomes the predominant GLUT4 uptake mechanism (Figure 10B). In this model, insulin does not specifically target GLUT4 endocytosis, but rather inhibits nystatin-sensitive uptake, the main basal GLUT4 uptake mechanism.

Numerous cargo proteins and lipids are internalized by nystatin-sensitive uptake pathways (Kirkham and Parton, 2005). Here we show that the nystatin-sensitive uptake mechanism is a novel target of insulin action. The inhibitory

Figure 6 GLUT4 is predominantly internalized by a nystatin-sensitive mechanism in basal adipocytes. (A) TR clathrin-dependent internalization is resistant to nystatin. Cells were treated for 1 h with 50 μ M nystatin or not treated (control) before the assay. The results are the mean $\pm$ s.d. from two independent experiments. The data were normalized to the control rate of each experiment. ns, not statistically significant. (B) Basal GLUT4 internalization is sensitive to nystatin whereas GLUT4 internalization upon insulin stimulation is resistant to nystatin. Cells were maintained in basal conditions or stimulated with 170 nM insulin for 30 min before addition of 50 μ M nystatin for 1 h. No nystatin was added to the control cells. The results are the mean $\pm$ s.d. from two independent experiments. The data were normalized to the control rate of wild-type GLUT4 of each experiment. (C) Nystatin and mutation of F^{5A} GLUT4 together completely inhibit basal GLUT4 internalization. Cells expressing wild-type GLUT4 or F^{5A}-GLUT4 were treated for 1 h with 50 μ M nystatin and GLUT4 internalization was measured. The data are the averages $\pm$ s.d. from two experiments.

effect of insulin on the nystatin-sensitive pathway will result in the acute accumulation on the plasma membrane of proteins and other components that are internalized through this pathway, and may thus have important physiologic consequences beyond modulation of GLUT4 internalization. For example, long-chain fatty acid transporters are localized to cholesterol-enriched domains of the plasma membrane (Ehehalt *et al*, 2006; Ring *et al*, 2006) and there is evidence that insulin increases the amount of the fatty acid transporters on the surface of cells (Stahl *et al*, 2002). Additionally, insulin signaling may affect lipid raft domains in a way that alters other functions of these domains, with the change in internalization being only one consequence of the effect.

The exact nature of basal GLUT4 endocytic mechanism remains to be determined. Although we cannot exclude the possibility that GLUT4 is internalized in basal adipocytes by an atypical clathrin-dependent pathway, the observations that basal uptake is sensitive to nystatin (unlike TR clathrin-dependent uptake) and independent of the AP-2 clathrin adaptor strongly suggest a clathrin-independent internalization mechanism. Several clathrin-independent endocytosis pathways have been described, all of which require

Figure 7 Insulin accelerates the nystatin-resistant uptake and inhibits the nystatin-sensitive uptake. (A) Insulin inhibits CT-B uptake. Results are the mean $\pm$ s.d. from two independent experiments (basal and insulin) or from one experiment with s.e.m. (nystatin). Within each experiment, the amount of CT-B taken up for 30 min was quantified for greater than 200 cells and normalized to the amount of CT-B bound to the plasma membrane at time 0. (B) Insulin increases TR internalization. The results are the mean $\pm$ s.d. from three independent experiments. The data were normalized to the basal rate of each experiment.

Figure 8 The suboptimal F^{5QI} endocytic signal is responsible for slow GLUT4 internalization in insulin-stimulated adipocytes. The endocytosis rate constants of wild type and F^{5Y} GLUT4 in basal and insulin-stimulated adipocytes are shown. The data are the means $\pm$ s.d. from three independent experiments.

free membrane cholesterol (reviewed in Naslavsky *et al*, 2004; Kirkham and Parton, 2005). One of these is the caveolin-dependent pathway. We did not observe any colocalization between GLUT4 and caveolin in adipocytes (unpublished observations), consistent with published reports (Parton *et al*, 2002; Shigematsu *et al*, 2003). The lack of colocalization, however, does not by itself rule out a role for caveolin in basal GLUT4 internalization, and a previous study using a caveolin mutant demonstrated a role for caveolin-1 in GLUT4 trafficking (Shigematsu *et al*, 2003). In addition to caveolin, other molecular components of the clathrin-independent uptake mechanism have been identified (e.g., Choudhury *et al*, 2006; Glebov *et al*, 2006). In this regard, we observed partial overlap in basal adipocytes between endocytosed CT-B and GLUT4 (Supplementary Figure 3), suggesting some

Figure 9 GLUT4 return-to-basal level after insulin withdrawal is a complex process involving the F⁵QQI and LL⁴⁹⁰ motifs as well as nystatin-sensitive pathways. Adipocytes were stimulated with insulin, the insulin removed and cells were fixed after different recovery times. The ratio of surface GLUT4 to total GLUT4 for each time point was fit to a single exponential decrease described by the following equation: $A_{\text{basal}} - (A_{\text{insulin}} \times \exp(-K_{\text{return}}t))$, where A represents the fraction of GLUT4 at the cell surface at either the basal (A_{basal}) or the insulin-stimulated (A_{insulin}) state, t is the time and K_{return} is the rate of return to the GLUT4 basal surface level. (A) The F⁵QQI and LL⁴⁹⁰ motifs are important for GLUT4 return-to-basal retention. The results are the mean $\pm$ s.d. from two (F⁵A, F⁵A/L⁴⁹⁰A) or three (wild type and L⁴⁹⁰A) independent experiments. Rates of GLUT4 return-to-basal level extracted from these plots are as follows: wild type: 0.04 min^{-1} ; F⁵A: 0.02 min^{-1} ; L⁴⁹⁰A: 0.02 min^{-1} and F⁵A/L⁴⁹⁰A: 0.003 min^{-1} . (B) Nystatin-sensitive pathways are implicated in GLUT4 return-to-basal. After 30 min insulin pre-stimulation, 50 $\mu\text{g/ml}$ nystatin was added for another 60 min before insulin removal. Nystatin was present during recovery time. The results are the mean $\pm$ s.d. from two (nystatin) or three (control) independent experiments. Rates of GLUT4 return-to-basal level extracted from these plots are as follows: control: 0.04 min^{-1} ; nystatin: 0.002 min^{-1} .

overlap in uptake mechanisms. However, CT-B is known to follow multiple endocytic mechanisms, which is an impediment to assessing the significance of colocalization between GLUT4 and CT-B. Additional studies are required to determine the molecular components of the predominant basal GLUT4 uptake.

Several observations indicated that GLUT4 follows a clathrin-dependent endocytosis pathway in insulin-stimulated adipocytes. In insulin-stimulated adipocytes, GLUT4 colocalizes with AP-2 and the TR in endocytic vesicles in the vicinity of the plasma membrane, GLUT4 endocytosis depends on the clathrin adaptor AP-2 and on the AP-2-interacting F⁵QQI motif, and GLUT4 endocytosis is resistant to nystatin treatment. Insulin accelerates the clathrin-depen-

dent internalization of TR, yet GLUT4 clathrin-dependent uptake in insulin-stimulated adipocytes is slow. The GLUT4 F⁵QQI motif resembles the canonical tyrosine-based endocytosis motifs (Bonifacino and Traub, 2003), which function to concentrate membrane proteins in clathrin-coated pits via interactions with AP-2. However, the presence of a phenylalanine rather than the more common tyrosine is striking, especially considering that a single nucleotide change transforms a phenylalanine codon to a tyrosine codon. The FQQI motif promotes a weaker interaction with the $\mu 2$ chain of AP-2 than the corresponding YQQI motif (Al-Hasani *et al*, 2002) and this results in a slower internalization in adipocytes (Figure 8). Thus, the conservation of a suboptimal internalization motif explains the slow internalization rate of GLUT4 in insulin-stimulated adipocytes.

There are conflicting reports in the literature regarding the mechanism of GLUT4 endocytosis. Some studies have suggested that GLUT4 is internalized via a clathrin-dependent mechanism (Nishimura *et al*, 1993; Parton *et al*, 2002) whereas other work implicated cholesterol-dependent mechanisms (Ros-Baro *et al*, 2001). We show here that GLUT4 is internalized through both pathways, with the predominant uptake mechanism depending on whether the adipocytes are resting or stimulated with insulin. Similarly, the relative contribution of the F⁵QQI and LL⁴⁹⁰ motifs to GLUT4 endocytosis was subject to debate (Corvera *et al*, 1994; Garippa *et al*, 1996; Al-Hasani *et al*, 2002; Govers *et al*, 2004). We found that the use of these motifs is tightly regulated. The FQQI motif has a minor role in basal internalization and major role in insulin internalization as well as in the return to basal retention following insulin withdrawal. The LL-based motif functions in the return to basal retention but does not regulate basal nor insulin internalization of GLUT4.

The return to basal retention measures the sum of a number of trafficking parameters and not solely GLUT4 endocytosis. One possible explanation for the observation that the LL-based motif affects return to basal retention without affecting internalization kinetics is that it is required at another step important for the transition. In this regard, James and co-workers (Govers *et al*, 2004) have recently shown a small increase in the basal recycling of an LL^{489,490}AA GLUT4 mutant. This effect in recycling kinetics may account for the slowing in the transition between stimulated and unstimulated states. Additional studies will be required to define the precise role of the LL-based motif in GLUT4 trafficking.

Our results uncover previously unexpected and complex mechanisms controlling GLUT4 internalization in adipocytes. Regulation of GLUT4 endocytosis has also been reported in muscle cells (Wijesekara *et al*, 2006). Thus, regulation of GLUT4 internalization occurs in the two main insulin-target tissues involved in the control of glucose homeostasis. Moreover, insulin inhibition of the cholesterol-dependent uptake pathway in adipocytes may have physiologic consequences beyond the regulation of GLUT4 trafficking.

Materials and methods

Cell culture and electroporation

3T3-L1 fibroblasts were differentiated and electroporated as previously described (Subtil *et al*, 2000; Zeigerer *et al*, 2002). Studies were performed on the day after electroporation, except for

Figure 10 Model for regulation of GLUT4 internalization. See text for discussion.

knockdown experiments in which cells were used 2 days after electroporation. For stable expression in 3T3-L1, pBabe puro/HA-GLUT4 retroviruses were produced in AmphoPack cells (ClonTech Mountain View, CA) and 3T3-L1 fibroblasts were infected and selected for growth in puromycin (Shewan *et al.*, 2000).

Plasmids, ligands and antibodies

The Stratagene (La Jolla, CA) QuickChange mutagenesis kit was used to introduce single base changes to mutate F⁵ and L⁴⁹⁰ in HA-GLUT4-GFP. The presence of the desired mutations was verified by sequencing (Cornell DNA sequencing facility, BRC, Ithaca, NY). The pSUPER- μ 2 plasmid was a gift of Dr P Benaroch (Institut Curie, Paris, France).

The HA.11 mouse monoclonal antibody was purified from ascites (Covance, Berkeley, CA) using a protein G affinity column (Amersham, Uppsala, Sweden). The saturating concentration of HA.11 was determined for each batch of purified antibody (Martin *et al.*, 2006). Anti-AP-2 α chain antibody was from ABR (Godlen, CO). All fluorescent secondary antibodies were from Jackson Immunolabs Inc. (West Grove, CA). Human transferrin (Sigma Inc., St Louis, MO) was purified by Sepharyl S-300 gel filtration and conjugated to fluorescent dyes following the manufacturer's instructions (Molecular Probes, Eugene, OR). B3/25 monoclonal antibody to the human TR was purified from hybridoma culture supernatant using a protein G affinity column (Amersham, Uppsala, Sweden). Alexa488 CT-B and anti-Alexa488 antibodies were from Molecular Probes (Eugene, OR).

Data acquisition and processing

Images were collected on a DMIRB inverted Leica microscope (Leica Microsystems, Deerfield, IL) using a $\times 40$ 1.25 NA oil-immersion objective or on a DMIRB inverted Zeiss microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) using a $\times 40$ 1.3 NA oil-immersion objective. Both microscopes were coupled to CCD 12-bit cameras (Princeton Instruments, West Chester, PA). 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. Fluorescence quantifications were carried out using Metamorph image processing software (Molecular Devices Corporation, Downingtown, PA) as described elsewhere (Lampson *et al.*, 2000, 2001; Karylowski *et al.*, 2004). Background was measured on similarly treated non-expressing cells, or, when not possible, on cells treated only with secondary antibodies. Background was subsequently subtracted from specific signal.

Endocytosis of GLUT4

Electroporated adipocytes were starved of serum for 2 h in DMEM medium with 20 mM HEPES and 20 mM sodium bicarbonate, pH 7.2 (DMEMBB) at 37°C in 5% CO₂, and stimulated (insulin) or not (basal) with 170 nM insulin for 30 min at 37°C in 5% CO₂. Cells were incubated in DMEMBB containing a saturating concentration

of HA.11 antibody for indicated pulse times. For internalization in the presence of nystatin, cells were stimulated or not for 30 min with insulin and treated for 1 h at 37°C with 50 μ g/ml nystatin. Nystatin and/or insulin were present during the HA.11 uptake when appropriate. For details concerning the uptake time courses, see Supplementary data. Cells were rapidly cooled, washed three times in ice-cold medium 2 (150 mM NaCl, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, 20 mM HEPES, pH 7.4) and fixed on ice for 6 min in phosphate-buffered saline (PBS)/3.7% formaldehyde. Surface-bound HA.11 was detected with a saturating concentration of Cy3-conjugated anti-mouse IgG antibody (15 μ g/ml in PBS/2% FBS). Cells were refixed for 5 min, and internalized HA.11 was revealed with Cy5-conjugated anti-mouse IgG antibodies (1.5 μ g/ml) in PBS/2% FBS and 250 μ M saponin. The slope of a plot of the ratio of internalized HA.11 (Cy5) to surface HA.11 (Cy3) is proportional to the internalization rate constant but is not the rate constant, as different fluorophores are used. This internalization rate parameter was normalized to the internalization rate parameter of wild type HA-GLUT4-GFP in basal adipocytes measured in the same experiment. To measure the internalization rate constant, the amount of GLUT4 on the cell surface was measured in separate dishes of identically treated cells, by staining surface-bound HA.11 antibodies with Cy5-conjugated antibodies. The internal (Cy5) to surface (Cy3) ratio for each time point was fit to a straight line, the slope of which is the internalization rate constant.

In this analysis, because the amount of internalized HA.11 at each time point is normalized for each cell to the amount of antibody bound to the surface, this endocytosis analysis is insensitive to changes in the amount of GLUT4 on cell surface that could result from changes in GLUT4 exocytosis/recycling. Mathematical description of the assay is available as Supplementary data.

GLUT4 return-to-basal retention after insulin removal

Cells were incubated in serum-free DMEMBB for 2 h, stimulated with 170 nM insulin for 30 min at 37°C and insulin was removed by alternate washes of neutral and acidic (pH 5.0) buffers (Subtil *et al.*, 2000). For experiments with nystatin, cells were prestimulated with insulin for 30 min and nystatin (50 μ g/ml) was then added for another 60 min before insulin removal. Cells were incubated in DMEMBB at 37°C in 5% CO₂ (with nystatin when appropriate) for various recovery times before fixation. At each time point, the surface-to-total HA-GLUT4-GFP was measured (Lampson *et al.*, 2001; Zeigerer *et al.*, 2002). This assay follows the change in surface GLUT4 over time, which is the result of a balance between exocytosis and endocytosis.

TR endocytosis

Endocytosis of the human TR expressed by electroporation in adipocytes was measured as previously described (Subtil *et al.*, 2000; Martin *et al.*, 2006) and details are provided on line as Supplementary data.

Cholera-toxin-B uptake

Differentiated adipocytes were starved of serum for 2 h at 37°C in DMEMBB, and pretreated with nystatin (50 µg/ml) for 1 h or with insulin (170 nM) for 30 min, or left untreated (basal). Cells were incubated on ice for 30 min with Alexa488-conjugated CT-B (2.5 µg/ml in MII buffer containing 1 mg/ml ovalbumin). Cells were washed twice with cold MII buffer and either fixed (surface Alexa488 CT-B at time 0), or switched back to 37°C by adding warm DMEMBB (supplemented with nystatin, insulin or nothing) for 30 min. Cells were fixed and the Alexa488 CT-B remaining on the cell surface was quenched using anti-Alexa488 antibodies (20 µg/ml in PBS/2% FBS). The non-quenchable fraction of surface-bound Alexa488 CT-B was estimated in parallel on samples that were fixed immediately after binding on ice. This value was subtracted from the value obtained for the 30 min uptake. The fluorescence of greater than 200 cells was measured and the uptake of Alexa488 CT-B was estimated by dividing the internalized Alexa488 CT-B by the surface Alexa488 CT-B at time 0.

AP-2 knockdown quantification

Two days after electroporation of adipocytes with the HA-GLUT4-GFP or the TR plasmid alone (control) or together with the pSUPER µ2 plasmid (knock down), cells were stained for AP-2 using the anti-AP-2 α chain antibody (1/1000 dilution in PBS/2% FBS/250 µM saponin) followed by Cy3-conjugated secondary antibodies. Background fluorescence was measured in separate dishes that were identically treated except being not incubated with anti-AP-2. Within each experiment, AP-2-associated fluorescence was normalized to the average value in control cells.

Total internal reflection fluorescence microscopy

Twenty-four hours after electroporation, adipocytes were incubated for 2 h in serum-free medium and stimulated or not with 170 nM insulin for 30 min. For AP-2 costaining, cells were fixed, permeabilized and AP-2 visualized in indirect immunofluorescence using Cy3-conjugated secondary antibodies. For transferrin costaining,

Alexa546-conjugated transferrin (10 µg/ml) was added in the media for 10 min before fixation.

The TIRF microscope used has been described previously (Moskowitz *et al*, 2003). A ×60 1.45 NA objective (Olympus America, Melville, NY) was used to perform 'prism-less' TIRF. The evanescent field decay length was 100–250 nm with this objective, with a pixel size of 112 × 112 nm² in the acquired images. Cells expressing HA-GLUT4-GFP were identified by GFP in epifluorescence mode. Both epifluorescence (GFP and Cy3) and TIRF (GFP) images of cells were acquired. For costaining with CT-B, adipocytes stably expressing HA-GLUT4 were used. Anti-HA.11 (saturated concentration) and Alexa-488-conjugated CT-B (10 µg/ml) were added to the media for 10 min before fixation. HA.11 was detected in indirect immunofluorescence using Cy3-conjugated secondary antibodies. Rabbit anti-Alexa488 was used to quench surface CT-B fluorescence. In this case, TIRF was used to detect intracellular CT-B within the TIRF zone and epifluorescence to detect internalized HA.11 antibodies.

MetaMorph software was used to process and overlay the images. Each image was scaled by eye to make the fluorescent structures easily visible. Overlay images are the direct results of already scaled GFP and Cy3 images and are not re-scaled.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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References

- Al-Hasani H, Kunamneni RK, Dawson K, Hinck CS, Muller-Wieland D, Cushman SW (2002) Roles of the N- and C-termini of GLUT4 in endocytosis. *J Cell Sci* **115**: 131–140
- Araki S, Yang J, Hashiramoto M, Tamori Y, Kasuga M, Holman GD (1996) Subcellular trafficking kinetics of GLUT4 mutated at the N- and C-terminal. *Biochem J* **315**: 153–159
- Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* **72**: 395–447
- Choudhury A, Marks DL, Proctor KM, Gould GW, Pagano RE (2006) Regulation of caveolar endocytosis by syntaxin 6-dependent delivery of membrane components to the cell surface. *Nat Cell Biol* **8**: 317–328
- Corvera S, Chawla A, Chakrabarti R, Joly M, Buxton J, Czech MP (1994) A double leucine within the GLUT4 glucose transporter COOH-terminal domain functions as an endocytosis signal. *J Cell Biol* **126**: 1625
- Czech MP, Buxton JM (1993) Insulin action on the internalization of the GLUT4 glucose transporter in isolated rat adipocytes. *J Biol Chem* **268**: 9187–9190
- Dugani CB, Klip A (2005) Glucose transporter 4: cycling, compartments and controversies. *EMBO Rep* **6**: 1137–1142
- Dugast M, Toussaint H, Dousset C, Benaroch P (2005) AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. *J Biol Chem* **280**: 19656–19664
- Ehehalt R, Fullekrug J, Pohl J, Ring A, Herrmann T, Stremmel W (2006) Translocation of long chain fatty acids across the plasma membrane—lipid rafts and fatty acid transport proteins. *Mol Cell Biochem* **284**: 135–140
- Garippa RJ, Johnson A, Park J, Petrush RL, McGraw TE (1996) The carboxyl terminus of GLUT4 contains a serine-leucine-leucine sequence that functions as a potent internalization motif in Chinese Hamster Ovary cells. *J Biol Chem* **271**: 20660–20668
- Garippa RJ, Judge TW, James DE, McGraw TE (1994) The amino terminus of GLUT4 functions as an internalization motif but not an intracellular retention signal when substituted for the transferrin receptor cytoplasmic domain. *J Cell Biol* **124**: 705–715
- Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* **8**: 46–54
- Govers R, Coster AC, James DE (2004) Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. *Mol Cell Biol* **24**: 6456–6466
- Huang F, Khvorova A, Marshall W, Sorkin A (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* **279**: 16657–16661
- Karylowski O, Zeigerer A, Cohen A, McGraw TE (2004) GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol Biol Cell* **15**: 870–882
- Kirkham M, Parton RG (2005) Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta* **1745**: 273–286
- Lampson MA, Racz A, Cushman SW, McGraw TE (2000) Demonstration of insulin-responsive trafficking of GLUT4 and vpr in fibroblasts. *J Cell Sci* **113** (Part 22): 4065–4076
- Lampson MA, Schmoranz J, Zeigerer A, Simon SM, McGraw TE (2001) Insulin-regulated release from the endosomal recycling compartment is regulated by budding of specialized vesicles. *Mol Biol Cell* **12**: 3489–3501
- Martin OJ, Lee A, McGraw TE (2006) GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *J Biol Chem* **281**: 484–490
- Mayor S, Presley JF, Maxfield FR (1993) Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J Cell Biol* **121**: 1257–1269
- Moskowitz HS, Heuser J, McGraw TE, Ryan TA (2003) Targeted chemical disruption of clathrin function in living cells. *Mol Biol Cell* **14**: 4437–4447

- Motley A, Bright NA, Seaman MN, Robinson MS (2003) Clathrin-mediated endocytosis in AP-2-depleted cells. *J Cell Biol* **162**: 909–918
- Naslavsky N, Weigert R, Donaldson JG (2004) Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol Biol Cell* **15**: 3542–3552
- Nishimura H, Zarnowski MJ, Simpson IA (1993) Glucose transporter recycling in rat adipose cells. Effects of potassium depletion. *J Biol Chem* **268**: 19246–19253
- Parton R, Molero J, Floetenmeyer M, Green K, James D (2002) Characterization of a distinct plasma membrane macrodomain in differentiated adipocytes. *J Biol Chem* **277**: p46769–p46778
- Piper RC, James DE, Slot JW, Puri C, Lawrence Jr JC (1993) GLUT4 phosphorylation and inhibition of glucose transport by dibutyl cAMP. *J Biol Chem* **268**: 16557–16563
- Ring A, Le Lay S, Pohl J, Verkade P, Stremmel W (2006) Caveolin-1 is required for fatty acid translocase (FAT/CD36) localization and function at the plasma membrane of mouse embryonic fibroblasts. *Biochim Biophys Acta* **1761**: 416–423
- Robinson LJ, Pang S, Harris DS, Heuser J, James DE (1992) Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP, insulin, and GTP gamma S and localization of GLUT4 to clathrin lattices. *J Cell Biol* **117**: 1181–1196
- Ros-Baro A, Lopez-Iglesias C, Peiro S, Bellido D, Palacin M, Zorzano A, Camps M (2001) Lipid rafts are required for GLUT4 internalization in adipose cells. *Proc Natl Acad Sci USA* **98**: 12050–12055
- Sandvig K, Spilsberg B, Lauvrak SU, Torgersen ML, Iversen TG, van Deurs B (2004) Pathways followed by protein toxins into cells. *Int J Med Microbiol* **293**: 483–490
- Schnitzer JE, Oh P, Pinney E, Allard J (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* **127**: 1217–1232
- Shewan A, Marsh B, Melvin D, Martin S, Gould G, James D (2000) The cytosolic C-terminus of the glucose transporter GLUT4 contains an acidic cluster endosomal targeting motif distal to the dileucine signal. [record supplied by publisher]. *Biochem J* **350**: p99–p107
- Shigematsu S, Watson R, Khan A, Pessin J (2003) The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4 [in process citation]. *J Biol Chem* **278**: p10683–p10690
- Stahl A, Evans JG, Pattel S, Hirsch D, Lodish HF (2002) Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev Cell* **2**: 477–488
- Subtil A, Lampson MA, Keller SR, McGraw TE (2000) Characterization of the insulin-regulated endocytic recycling mechanism in 3T3-L1 adipocytes using a novel reporter molecule. *J Biol Chem* **275**: 4787–4795
- Verhey KJ, Yeh JJ, Birnbaum MJ (1995) Distinct signals in the GLUT4 glucose transporter for internalization and for targeting to an insulin-responsive compartment. *J Cell Biol* **130**: 1071–1079
- Watson RT, Kanzaki M, Pessin JE (2004) Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocr Rev* **25**: 177–204
- Wijesekara N, Tung A, Thong FS, Klip A (2006) Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis, independently of AMPK. *Am J Physiol Endocrinol Metab* **290**: E1276–E1286
- Wiley HS, Cunningham DD (1982) The endocytotic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. *J Biol Chem* **257**: 4222–4229
- Zeigerer A, Lampson M, Karylowski O, Sabatini D, Adesnik M, Ren M, McGraw T (2002) GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. *Mol Biol Cell* **13**: p2421–p2435
- Zeigerer A, McBrayer MK, McGraw TE (2004) Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. *Mol Biol Cell* **15**: 4406–4415