

Rab10, a Target of the AS160 Rab GAP, Is Required for Insulin-Stimulated Translocation of GLUT4 to the Adipocyte Plasma Membrane

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SUMMARY

GLUT4 trafficking to the plasma membrane of muscle and fat cells is regulated by insulin. An important component of insulin-regulated GLUT4 distribution is the Akt substrate AS160 rab GTPase-activating protein. Here we show that Rab10 functions as a downstream target of AS160 in the insulin-signaling pathway that regulates GLUT4 translocation in adipocytes. Overexpression of a mutant of Rab10 defective for GTP hydrolysis increased GLUT4 on the surface of basal adipocytes. Rab10 knockdown resulted in an attenuation of insulin-induced GLUT4 redistribution to the plasma membrane and a concomitant 2-fold decrease in GLUT4 exocytosis rate. Re-expression of a wild-type Rab10 restored normal GLUT4 translocation. The basal increase in plasma-membrane GLUT4 due to AS160 knockdown was partially blocked by knocking down Rab10 in the same cells, further indicating that Rab10 is a target of AS160 and a positive regulator of GLUT4 trafficking to the cell surface upon insulin stimulation.

INTRODUCTION

Insulin regulates the uptake of glucose into adipose and muscle tissues by regulating the amount of the GLUT4 glucose transporter in the plasma membrane (Dugani and Klip, 2005; Watson and Pessin, 2006). This process is important for the regulation of whole-body glucose homeostasis. GLUT4 is retained intracellularly in unstimulated adipocytes by a dynamic bipartite process involving slow exocytosis and rapid endocytosis. Insulin stimulates GLUT4 exocytosis and slows its endocytosis, resulting in a net accumulation of GLUT4 in the plasma membrane.

One of the unanswered questions in the field is how, at a molecular level, insulin signals to GLUT4 trafficking. Insulin signaling to GLUT4 is complex (Saltiel and Pessin, 2003; Watson et al., 2004). The main signal transduction pathway involves insulin activation of the serine/threonine kinase Akt (PKB) (e.g., Bae et al., 2003; Whiteman et al., 2002), although there is evidence for the involvement of atypical protein kinase C isoforms (Sajan et al., 2006) as well as activation of TC10, a member of the Rho family of small GTPases (Chang et al., 2007). Recently, a substrate of Akt that is along the signal transduction pathway to GLUT4, known as AS160, has been identified (Sano et al., 2003). AS160 has a rab GTPase-activating protein (GAP) domain that is required for its effects on GLUT4 trafficking (Eguez et al., 2005; Larance et al., 2005; Sano et al., 2003). The rab GTPases are key regulators of membrane trafficking (Grosshans et al., 2006). Rab proteins cycle between GDP- and GTP-bound forms, and the GTP form is typically considered the active form. Accessory factors regulate this steady state and thereby determine whether a rab is active or inactive. A rab GAP interacts with its substrate rabs and stimulates the intrinsic GTPase activity of the rab, increasing the portion of the rab in the inactive GDP form.

One model of the role of AS160 in GLUT4 trafficking is that, in basal adipocytes, AS160 is an active rab GAP that functions to maintain one or more rab proteins in the inactive GDP form. Akt phosphorylation of AS160 inhibits the GAP activity, leading to an increase in the active GTP-bound form of the AS160 target rab (or rabs). Key aspects of this model are supported by the results of studies of 3T3-L1 adipocytes in which AS160 has been knocked down, as well as by studies of the effects on GLUT4 behavior of overexpressing AS160 mutants (Eguez et al., 2005; Larance et al., 2005; Sano et al., 2003; Zeigerer et al., 2004). Specifically, AS160 knockdown results in an acceleration of basal GLUT4 exocytosis and a concomitant increase of GLUT4 in the plasma membrane (Eguez et al., 2005; Larance et al., 2005). The GAP activity of AS160 is essential for basal GLUT4 retention since an AS160 mutant with an inactive GAP domain is unable to

rescue the AS160 knockdown phenotype (Eguez et al., 2005).

The results from the previous studies support the hypothesis that AS160 inhibits basal GLUT4 exocytosis by negatively regulating one or more rab proteins. Here we report that Rab10, which is found in GLUT4 vesicles (Larance et al., 2005; Miinea et al., 2005) and is a substrate for the GAP domain of AS160 (Miinea et al., 2005), is required for insulin signaling to GLUT4 translocation. Overexpression of a GTP-mimic form of Rab10 (Rab10-QL) partially induces a redistribution of GLUT4 to the plasma membrane of basal adipocytes, whereas siRNA-mediated knockdown of Rab10 attenuates translocation by partially abrogating insulin stimulation of GLUT4 exocytosis. The effect of the knockdown on GLUT4 is rescued by re-expression of wild-type Rab10, indicating that the effects are due to the loss of Rab10. Knockdown of Rab10 in AS160 knockdown adipocytes partially restores basal GLUT4 retention, providing direct evidence for the proposal that the phenotype of AS160 knockdown adipocytes is due to increased Rab10 in the GTP-bound form and therefore that one target of AS160 action is Rab10. Insulin, through activation of Akt, increases GLUT4-containing vesicle exocytosis, in part by stimulating pre-fusion vesicle docking/recruitment to the plasma membrane (Gonzalez and McGraw, 2006). Rab10 knockdown inhibits insulin regulation of GLUT4-containing vesicle docking/recruitment, consistent with Rab10 being downstream of Akt action. Together, these data argue that Rab10 is a substrate of AS160 required for insulin-stimulated redistribution of GLUT4 to the plasma membrane and thereby suggest that it has a role in the regulation of glucose homeostasis.

RESULTS

Rab10-QL Induces a Redistribution of GLUT4 to the Plasma Membrane of Basal Adipocytes

In a recent study, we have found that the AS160 GAP domain has activity toward Rab10 (Miinea et al., 2005). To investigate whether Rab10 is a relevant target of AS160 action required for insulin-stimulated translocation of GLUT4 to the plasma membrane of adipocytes, we examined the effect of overexpressing a mutant Rab10 that mimics GTP-bound Rab10 (active form). This Rab10 mutant is one in which a leucine is substituted for glutamine at position 68 (designated Rab10-QL). The corresponding Q/L mutation in other rab proteins inhibits the intrinsic GTPase activity and thereby results in an increase in the rab GTP-bound form (e.g., Stenmark et al., 1994). Rab10 cDNAs were coexpressed in 3T3-L1 adipocytes with HA-GLUT4-GFP, a reporter used to characterize GLUT4 behavior, by electroporation (Zeigerer et al., 2002). Overexpression of the Rab10-QL mutant resulted in a greater than 2-fold increase of HA-GLUT4-GFP in the plasma membrane of basal adipocytes (Figures 1A and 1B). Overexpression of wild-type Rab10 resulted in a significantly smaller increase of plasma-membrane GLUT4, demonstrating that the active form of Rab10 has a more potent

effect on GLUT4 in basal adipocytes (Figures 1A and 1B). Overexpression of wild-type Rab10 or Rab10-QL did not have a significant effect on the amount of GLUT4 in the plasma membrane after insulin stimulation (Figure 1C), showing that the effects of Rab10-QL are specific to basal adipocytes. We were unable to determine the effect of overexpression of a Rab10 mutated to preferentially bind GDP (Rab10-T²³N) because this mutant was not expressed in adipocytes, probably because of a reduced stability (Schuck et al., 2007).

The transferrin receptor is constitutively recycled between the plasma membrane and the interior of cells by the general endosomal recycling system. Previous studies have documented that AS160 does not have a role in regulating the trafficking of the transferrin receptor in adipocytes. Based on those findings, Rab10 would not be anticipated to have a role in the regulation of transferrin receptor behavior in adipocytes. Consistent with that proposal, overexpression of Rab10-QL did not alter the behavior of the transferrin receptor in basal or insulin-stimulated adipocytes (Figure 1D). These data establish that the effect of Rab10-QL on GLUT4 is not due to a perturbation of the general membrane recycling pathway.

Previous studies have localized Rab10 to GLUT4-containing vesicles with biochemical methods (Larance et al., 2005; Miinea et al., 2005). Our Rab10 antibody does not work in immunofluorescence, and we were therefore unable to localize endogenous Rab10 in 3T3-L1 adipocytes by immunofluorescence. However, the exogenously expressed FLAG-tagged Rab10 localizes with HA-GLUT4-GFP in the perinuclear region as well as in puncta distributed throughout the cytoplasm of adipocytes (Figure 1E). The expression of the QL mutant does not markedly alter the morphology of intracellular compartments containing HA-GLUT4-GFP. This partial colocalization with GLUT4 is consistent with the previous biochemical studies and with Rab10 having a role in GLUT4 vesicle redistribution to the plasma membrane.

Rab10 Knockdown Blunts the Insulin-Stimulated Redistribution of GLUT4

The results of the above studies examining the effect of overexpressing the constitutively active Rab10-QL mutant on GLUT4 behavior in basal adipocytes support the hypotheses that Rab10 is downstream of AS160 and that AS160 is a negative regulator of Rab10. As an alternative means of investigating the role of Rab10 in insulin regulation of GLUT4 trafficking, we used siRNA methods to knock down Rab10. 3T3-L1 fibroblasts were infected with pSIREN retroviruses encoding sequences that target Rab10. The infected cells were differentiated and the knockdown of Rab10 was determined by western blotting. Two targeting sequences, 5'-TTCGACGATGCCCTCAA-3' (KD(87)) and 5'-GCATCATGCTAGTGTATGA-3' (KD(251)), were found to reduce Rab10 protein expression by approximately 70% and 90%, respectively (Figure 2A). The knockdown of Rab10 did not affect the expression of Rab8, Rab4, or Rab11 (Figures 2A and 2B). Based on visual inspection of the adipocytes and the expression of

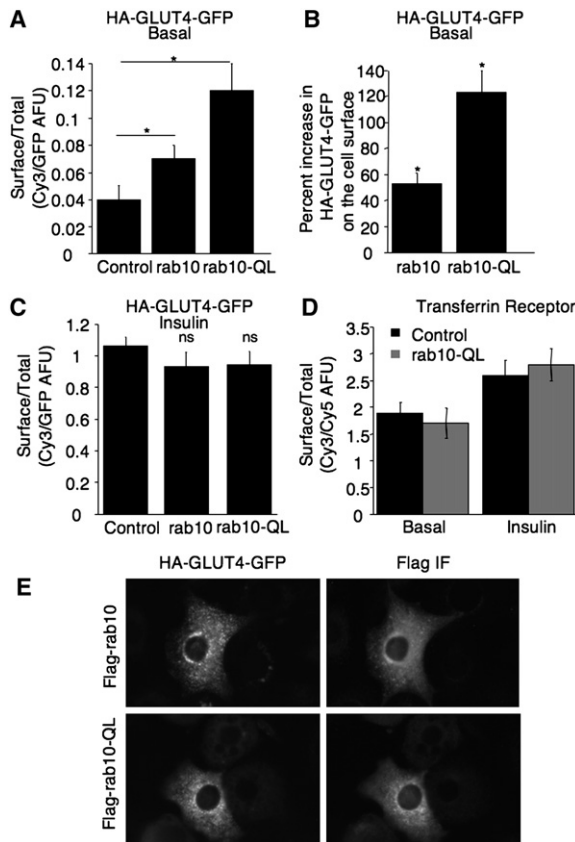


Figure 1. Overexpression of Rab10-QL Induces a Redistribution of GLUT4 to the Plasma Membrane Independent of Insulin

(A) HA-GLUT4-GFP in the plasma membrane of basal adipocytes. Cells were electroporated with cDNAs for HA-GLUT4-GFP alone (control), HA-GLUT4-GFP and wild-type FLAG-Rab10, or HA-GLUT4-GFP and FLAG-Rab10-QL mutant. The data are from a single experiment. The average surface-to-total ratio of more than 20 cells per condition \pm SEM is shown. Coexpression of rab constructs with HA-GLUT4-GFP was confirmed by anti-FLAG epitope indirect immunofluorescence. * $p < 0.05$ by two-tailed, paired Student's *t* test.

(B) The average \pm SEM of eight experiments showing the effect of Rab10-QL overexpression on HA-GLUT4-GFP distribution to the plasma membrane of basal adipocytes compared to overexpression of wild-type Rab10 (average \pm SEM of three experiments). * $p < 0.05$ by two-tailed, paired Student's *t* test compared to control.

(C) Data from a representative experiment measuring HA-GLUT4-GFP in the plasma membrane of insulin-stimulated adipocytes. The average surface-to-total ratio of more than 20 cells per condition \pm SEM is shown. Neither Rab10 nor Rab10-QL had a significant effect on the amount of HA-GLUT4-GFP in the plasma membrane of insulin-stimulated cells in this experiment or when data from six experiments for Rab10-QL and two experiments for wild-type Rab10 were averaged. Cells were stimulated with 170 nM insulin for 30 min prior to measuring HA-GLUT4-GFP in the plasma membrane. ns, not statistically different from control.

(D) Average \pm SD of two experiments examining the effect of overexpression of Rab10-QL on the distribution of the transferrin receptor. Cells were stimulated with 170 nM insulin for 30 min prior to measuring transferrin receptor in the plasma membrane.

(E) Colocalization of HA-GLUT4-GFP with wild-type FLAG-Rab10 or FLAG-Rab10-QL mutant. The distribution of HA-GLUT4-GFP was determined by GFP fluorescence, and the distribution of Rab10

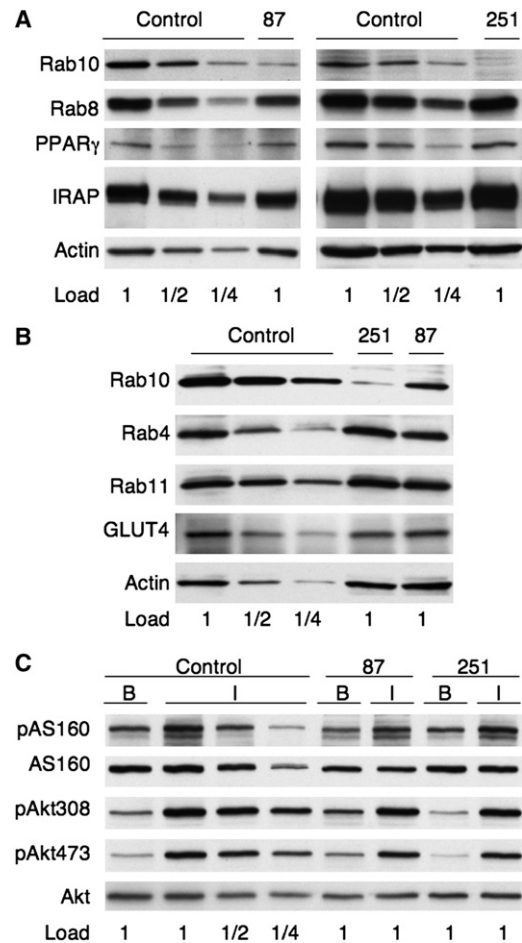


Figure 2. Rab10 Knockdown Does Not Affect Differentiation or Insulin Activation of Akt in 3T3-L1 Adipocytes

(A) SDS lysates of cells expressing the control shRNA or the Rab10 KD(87) (left) or KD(251) (right) shRNA were immunoblotted for Rab10 and Rab8, the differentiation markers PPAR γ and IRAP, and actin as a loading control.

(B) SDS lysates of cells expressing the control shRNA or the Rab10 KD(251) (left) or KD(87) (right) shRNA were immunoblotted for Rab10, Rab4, and Rab11; GLUT4; and actin as a loading control.

(C) SDS lysates from basal (B) and insulin-treated (170 nM, 15 min) (I) cells as described in (A) were immunoblotted for AS160 and Akt expression and phosphorylation. Phosphorylated AS160 (pAS160) was detected with the phospho-Akt substrate antibody. The protein concentration of the samples was determined by a precipitating Lowry assay, and the 1 \times load in each case contained the same amount of protein. The slightly lower signal for pAkt473 in the basal state for Rab10 KD(251) compared to the control and KD(87) was variable; with a second set of samples from another plating of cells, there was no difference between the pAkt473 signal in the three samples.

differentiation-specific proteins, the transcription factor PPAR γ , the insulin-regulated aminopeptidase (IRAP), and GLUT4, Rab10 knockdown did not affect the differentiation of preadipocytes to adipocytes (Figures 2A and 2B).

constructs by indirect immunofluorescence using an anti-FLAG epitope antibody. The images are epifluorescence acquired with a 63 \times objective.

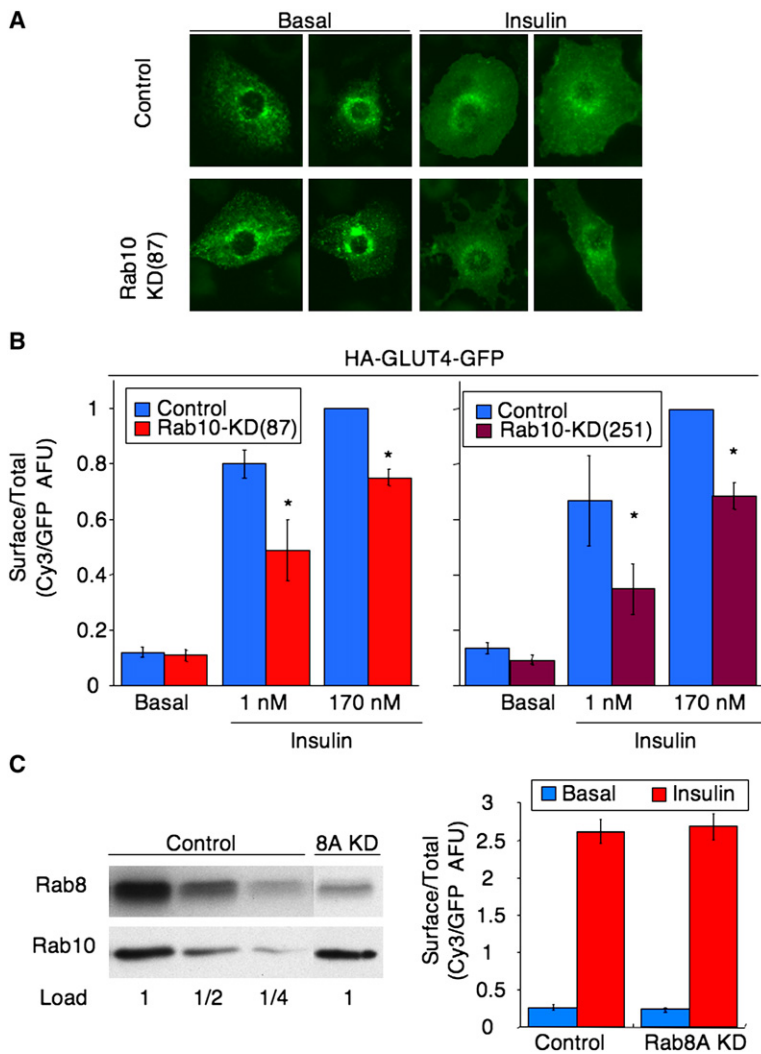


Figure 3. Rab10 Is Required for Full Translocation of GLUT4 to the Plasma Membrane

(A) Localization of HA-GLUT4-GFP in adipocytes infected with a retrovirus harboring a shRNA sequence that does not target any mouse gene (control) or shRNA sequences targeting Rab10 KD(87). GFP fluorescence detected by epifluorescence is shown for two cells per line per condition. Basal cells were serum starved for 2 hr, and insulin-stimulated cells were serum starved for 2 hr followed by 30 min incubation in 170 nM insulin.

(B) Adipocytes from 3T3-L1 cells infected with a control retrovirus or retroviruses with shRNA sequences targeting Rab10 KD(87) and KD(251) were electroporated with HA-GLUT4-GFP, and the distribution of HA-GLUT4-GFP was determined 24 hr after electroporation. The data are averages \pm SEM of independent experiments (KD(87): basal $n = 6$, 1 nM $n = 3$, 170 nM $n = 9$; KD(251): basal and 1 nM $n = 2$, 170 nM $n = 4$). * $p < 0.05$. Basal cells were serum starved for 2 hr, and insulin-stimulated cells were serum starved for 2 hr followed by 30 min incubation in insulin.

(C) Adipocytes from 3T3-L1 cells infected with a control retrovirus or a retrovirus with a shRNA sequence targeting Rab8A (5'-GGAATAAGTG TGATGTGAA-3') were electroporated with HA-GLUT4-GFP and analyzed after 24 hr. SDS lysates of the cells containing equal amounts of protein were immunoblotted for Rab8A and Rab10, as shown in the left panel. The blots for Rab8A and Rab10 were performed using the same SDS samples, and all lanes on each blot were from the same immunoblot; the right lane (Rab8A KD) appears pasted because several intervening lanes irrelevant to the point of the figure have been removed. The distribution of HA-GLUT4-GFP is shown in the right panel. The data are from a representative experiment. The average surface-to-total ratio of more than 20 cells per condition \pm SEM is shown. Insulin (170 nM) treatment was for 30 min.

In Rab10 knockdown cells, as in control adipocytes, HA-GLUT4-GFP was localized predominantly to the perinuclear region (Figure 3A). In both control and Rab10 knockdown insulin-stimulated adipocytes, there was an increase in punctate GFP fluorescence throughout the cytosol as well as an increase in diffuse fluorescence, which likely reflects increased HA-GLUT4-GFP in the plasma membrane (Figure 3A). Thus, based on a qualitative assessment, knockdown of Rab10 did not result in gross alterations in GLUT4 behavior.

A quantitative analysis demonstrated that Rab10 knockdown blunted the insulin-stimulated translocation of GLUT4 to the plasma membrane by about 30% without affecting the basal retention (Figure 3B). The effect of the knockdown was observed at insulin concentrations that induce submaximal as well as maximal translocation (Figure 3B). We did not observe any significant differences

between Rab10 KD(87) and KD(251) adipocytes. The blunting of GLUT4 translocation by Rab10 knockdown was not due to a gross alteration in insulin signal transduction since insulin-stimulated phosphorylation of Akt and AS160 was not altered in the knockdown cells (Figure 2C). Furthermore, knockdown of Rab8A, another substrate of the AS160 GAP domain (Miinea et al., 2005), by 70% did not affect insulin-stimulated translocation of GLUT4 to the plasma membrane (Figure 3C).

The Rab10 knockdown in the 3T3-L1 adipocytes caused a decrease of approximately 10% in insulin-stimulated hexose transport, a reduction that was not statistically significant (see Figure S1 in the Supplemental Data available with this article online). As shown recently, about 50% of the insulin-stimulated hexose transport in 3T3-L1 adipocytes is due to GLUT1 redistribution (Liao et al., 2006). Therefore, the expected 30% reduction in

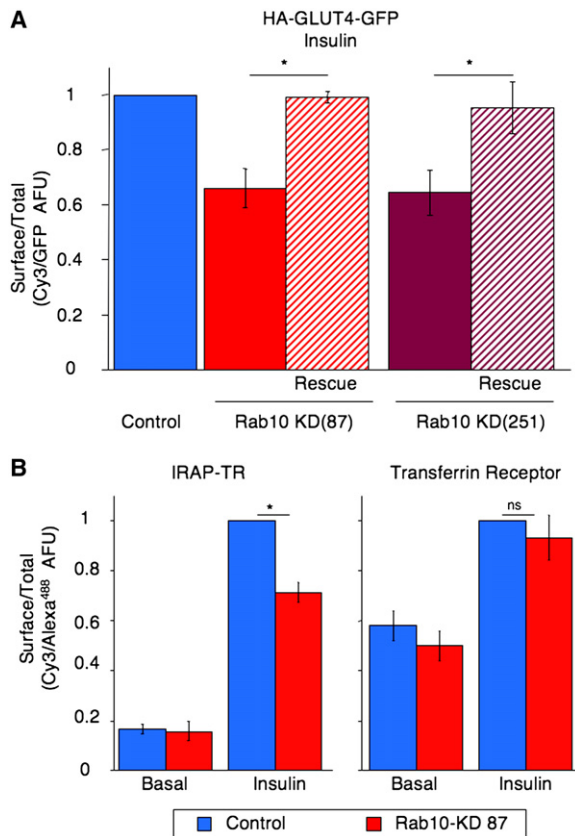


Figure 4. The Effects of Rab10 Knockdown Are Specific to GLUT4 and IRAP

(A) Rab10 KD(87) and KD(251) adipocytes were electroporated with HA-GLUT4-GFP or with HA-GLUT4-GFP and Rab10 constructs in which several wobble positions of the codons in the siRNA target sequence were mutated to make the Rab10 resistant to knockdown (rescue). Data are the averages \pm SD of three experiments. * $p < 0.05$ by paired Student's *t* test. Insulin treatment was 170 nM for 30 min. Expression of the wobbled wild-type Rab10 was confirmed by anti-FLAG epitope indirect immunofluorescence.

(B) Distributions of IRAP-TR and the transferrin receptor between the plasma membrane and interior of adipocytes expressing Rab10 KD(87) or control shRNA. The data are the averages \pm SD of three experiments. Cells were incubated in 170 nM insulin for 30 min. * $p < 0.05$ by paired Student's *t* test. ns, not statistically different from control.

cell-surface GLUT4 induced by the Rab10 knockdown (Figure 3B) would be expected to reduce glucose transport by only 15%.

Transient Re-expression of Rab10 Rescues the Effect of Rab10 Knockdown on Insulin-Stimulated GLUT4 Translocation

To establish that the phenotype of the Rab10 knockdown cells was specific to the reduction in Rab10, we determined the effect of re-expressing Rab10 in these cells. We created Rab10 constructs in which the wobble positions of several codons within the target siRNA target sequences were mutated, making these Rab10 mRNAs resistant to knockdown. Re-expression of the knockdown-resistant FLAG-Rab10 was established by anti-

FLAG indirect immunofluorescence. The resistant FLAG-Rab10 was expressed in the knockdown adipocytes at a level similar to in the control cells (data not shown).

Re-expression of Rab10 in the knockdown adipocytes restored insulin-stimulated GLUT4 translocation to control levels (Figure 4A). These data indicate that blunted redistribution of GLUT4 results from the Rab10 knockdown, providing additional evidence that Rab10 has a role in the regulation of GLUT4 trafficking. In addition, the fact that the altered phenotype of the knockdown adipocytes can be rescued by transient re-expression of Rab10 shows that the phenotype is not due to an alteration in the differentiation of the adipocytes.

Rab10 Knockdown Blunts the Insulin-Stimulated Redistribution of IRAP, but Not of the Transferrin Receptor

The other known cargo protein of the insulin-regulated GLUT4 trafficking pathway is IRAP, a transmembrane aminopeptidase (Garza and Birnbaum, 2000; Kandror and Pilch, 1994; Keller et al., 1995; Ross et al., 1996). To determine whether Rab10 knockdown affects the insulin-regulated trafficking pathway or whether the effects are specific to GLUT4, we examined the behavior of an established reporter of IRAP trafficking, IRAP-TR (Subtil et al., 2000; Zeigerer et al., 2002). The knockdown of Rab10 blunted insulin-stimulated translocation of IRAP-TR to the plasma membrane, indicating that loss of Rab10 affects the insulin-regulated pathway and is not restricted specifically to GLUT4 (Figure 4B).

There is substantial overlap between the general endocytic trafficking pathway and the insulin-regulated GLUT4 pathway (Karylowski et al., 2004; Martin et al., 1996). To determine whether the Rab10 knockdown affects both pathways, we examined the behavior of the transferrin receptor. The distribution of the transferrin receptor between the interior and surface of basal and insulin-stimulated adipocytes was unaffected by knockdown of Rab10 (Figure 4B). These data show that Rab10 knockdown does not affect the general recycling pathway.

Knockdown of Rab10 Partially Restores GLUT4 Retention in Adipocytes Lacking AS160

Knockdown of AS160 results in an increase of GLUT4 in the plasma membrane of basal adipocytes (Eguez et al., 2005; Larance et al., 2005). Re-expression of a GAP-inactive mutant of AS160 cannot rescue the AS160 knockdown phenotype, indicating that the active AS160 GAP is required for basal retention (Eguez et al., 2005). One interpretation of these data is that the increase of GLUT4 in the plasma membrane of basal AS160 knockdown adipocytes is due to an inappropriate activation of an AS160-targeted rab protein. To determine whether Rab10 is involved in the increase of GLUT4 in the plasma membrane of basal AS160 knockdown adipocytes, we knocked down Rab10 in these adipocytes with a mixture of RNAi sequences prepared in vitro by dicer enzymatic treatment of Rab10 mRNA (Myers et al., 2003, 2006). The Rab10 siRNA pool knocked down Rab10 by 60% in wild-type

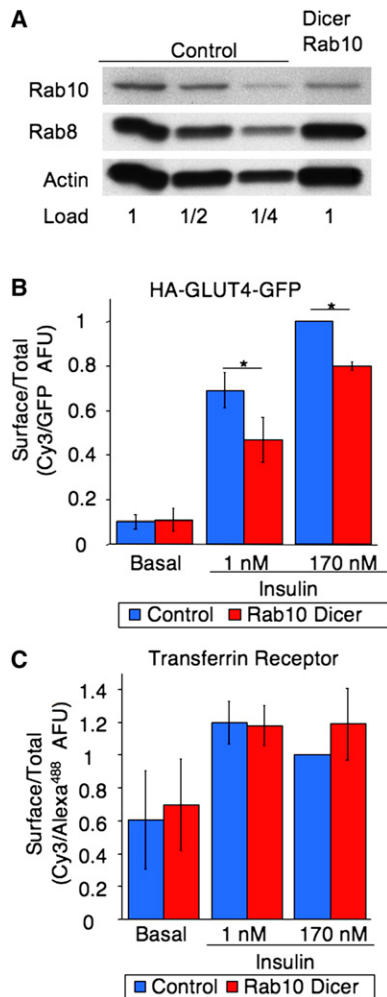


Figure 5. Transient Knockdown of Rab10 in Adipocytes Has the Same Effect on GLUT4 as Stable Rab10 Knockdown, without an Effect on the Transferrin Receptor

(A) SDS lysates of control cells and cells transfected with the Rab10 dicer pool were immunoblotted for Rab10, Rab8, and actin as a loading control.

(B) 3T3-L1 adipocytes were coelectroporated with pooled Rab10 dicer siRNA and HA-GLUT4-GFP cDNA or with HA-GLUT4-GFP cDNA alone (control). The behavior of HA-GLUT4-GFP was examined 48 hr after electroporation. Insulin incubations were for 30 min. Data are the averages \pm SEM of five experiments. * $p < 0.05$ by paired Student's *t* test.

(C) Adipocytes were coelectroporated with Rab10 dicer siRNA and human transferrin receptor cDNA or with human transferrin receptor cDNA alone (control). The behavior of the transferrin receptor was examined 48 hr after electroporation. Insulin incubations were for 30 min. Data are the averages of two experiments \pm SD.

and AS160 knockdown adipocytes within 48 hr of electroporation (Figure 5A and data not shown) without any effect on Rab8 expression (Figure 5A). We found that more than 90% of adipocytes take up a fluorescent siRNA, and therefore, the 60% Rab10 knockdown reflects the average reduction of Rab10 in most of the cells induced by the dicer siRNA (data not shown).

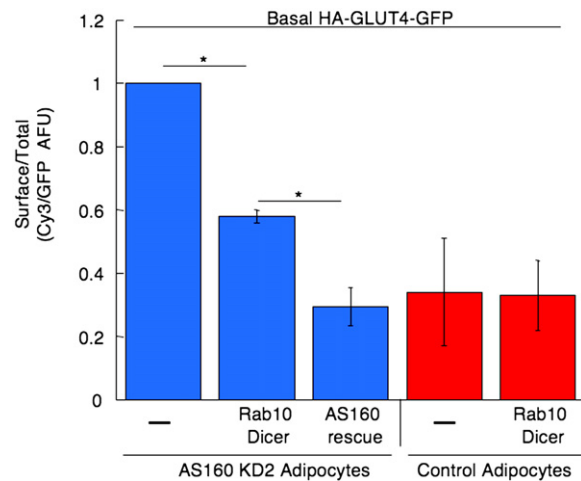


Figure 6. Transient Knockdown of Rab10 in AS160 Knock-down Adipocytes Partially Restores Basal Retention of GLUT4

Adipocytes from 3T3-L1 cells infected with a retrovirus harboring a shRNA sequence that targets AS160 (AS160 KD2; Eguez et al., 2005) were electroporated with HA-GLUT4-GFP cDNA, HA-GLUT4-GFP cDNA and Rab10 dicer siRNA, or knockdown-resistant AS160 cDNA (to completely rescue the AS160 knockdown; Eguez et al., 2005). The distribution of HA-GLUT4-GFP in basal adipocytes was measured 48 hr after electroporation. Data are the averages of four experiments \pm SEM. * $p < 0.05$ by paired Student's *t* test. For comparison, the basal retention of GLUT4 in control adipocytes and control adipocytes electroporated with Rab10 dicer siRNA is shown.

The effects of the Rab10 knockdown by the dicer pool were similar to the effects of the stable knockdown using the pSIREN system: insulin-stimulated translocation of GLUT4 to the plasma membrane was blunted, whereas the behavior of the transferrin receptor was unaffected (Figures 5B and 5C). Electroporation of the adipocytes with a diced siRNA pool against firefly luciferase did not affect HA-GLUT4-GFP in the plasma membrane in either basal or insulin-stimulated adipocytes (data not shown).

Transient knockdown of Rab10 with the dicer pool in adipocytes in which AS160 was constitutively knocked down (Eguez et al., 2005) reduced the basal amount of GLUT4 in the plasma membrane (Figure 6). However, it did not restore GLUT4 retention to the level achieved by transiently re-expressed AS160, which completely restores basal GLUT4 retention (Figure 6). The incomplete restoration may be due to the incomplete dicer knockdown of Rab10 (Figure 5A). Nevertheless, these data are consistent with the hypothesis that Rab10 is a downstream effector of AS160 involved in the regulation of GLUT4 trafficking.

Knockdown of Rab10 Blunts Insulin Stimulation of GLUT4 Exocytosis

AS160 is a negative regulator of GLUT4 exocytosis (Eguez et al., 2005). Thus, if Rab10 is downstream of AS160, then the blunting of insulin-stimulated GLUT4 translocation in Rab10 knockdown adipocytes should result from slower

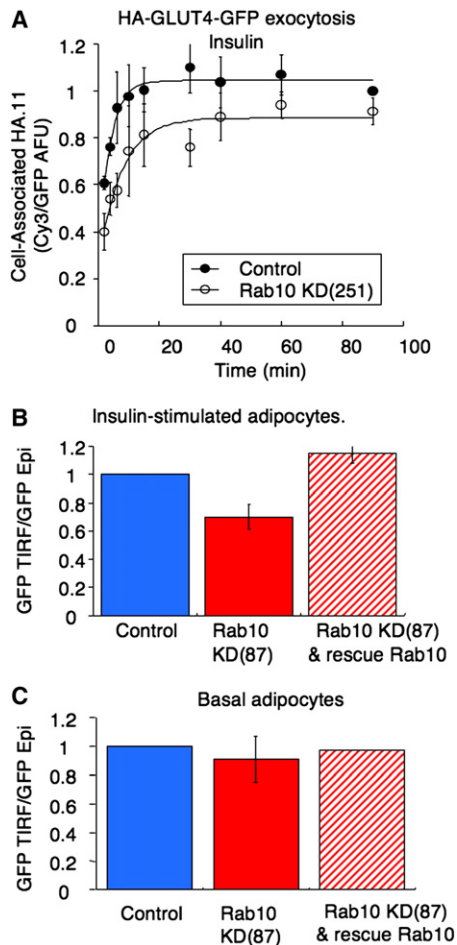


Figure 7. Rab10 Knockdown Attenuates Insulin Stimulation of GLUT4 Exocytosis by Attenuating Insulin-Stimulated Translocation of GLUT4 Measured by TIRF Microscopy

(A) HA-GLUT4-GFP exocytosis measured in adipocytes expressing control or Rab10 KD(251) shRNA stimulated with insulin. Cells were prestimulated with 170 nM insulin for 30 min, and 170 nM insulin was maintained in the incubation medium for the duration of the experiment. The data are the averages of three experiments \pm SD. The values at each time point within each experiment were normalized to the control value at 90 min. The data are fit to the following equation: cell-associated HA.11_t = cell-associated HA.11_{plateau} - (intracellular HA.11_{t=0} \times $\exp(-K_{\text{exocytosis}} \times t)$).

(B and C) Control and Rab10 KD(87) adipocytes were transfected with HA-GLUT4-GFP, and Rab10 KD(87) adipocytes were also transfected with HA-GLUT4-GFP and wobbled wild-type Rab10, which is resistant to the knockdown. The amount of GLUT4 in the evanescent field was measured in GFP fluorescence following a 30 min incubation with 170 nM insulin (B) or in basal conditions (C). Total GFP signal in the cells was determined in the epifluorescence mode of the microscope. The TIRF/epi fluorescence ratio per cell was calculated for at least 20 cells per condition per experiment. The data are the averages of two experiments \pm SD. In each experiment, the TIRF/epi ratio was normalized to the control value. As described in Gonzalez and McGraw (2006), the TIRF/epi ratio for the control in the insulin-stimulated state was approximately 2.5 times larger than that for the control in the basal state.

GLUT4 exocytosis in the insulin-stimulated Rab10 knockdown adipocytes. To test this hypothesis, the steady-state insulin-stimulated exocytosis kinetics of

HA-GLUT4-GFP in control and Rab10 knockdown cells were compared using an anti-HA antibody (HA.11) uptake assay (Karylowski et al., 2004). In control insulin-stimulated adipocytes, about 60% of HA-GLUT4-GFP was in the plasma membrane and therefore was immediately bound by the HA.11 (Figure 7A). The cell-associated HA.11 increases with time as internal HA-GLUT4-GFP cycles to the plasma membrane, where it is bound by HA.11 in the medium, reaching a plateau when all HA-GLUT4-GFP is bound by HA.11. In control insulin-stimulated adipocytes, the half-time for GLUT4 cycling to the plasma membrane was about 5 min (Figure 7A). In insulin-stimulated Rab10 knockdown adipocytes, less HA-GLUT4-GFP was in the plasma membrane (about 40%), and the remaining intracellular HA-GLUT4-GFP recycled more slowly than in control cells, with a half-time of about 10 min (Figure 7A). Thus, Rab10 knockdown resulted in a near 2-fold reduction in insulin-stimulated exocytosis of GLUT4. In addition to the slower recycling, the plateau in the Rab10 knockdown was reduced by about 10% compared to control cells.

Rab10 Knockdown Blunts Insulin-Stimulated Redistribution of GLUT4 to within 250 Nanometers of the Plasma Membrane

Akt-mediated signaling regulates one or more pre-fusion steps consisting of GLUT4-containing vesicle recruitment and/or docking with the plasma membrane (Bai et al., 2007; Gonzalez and McGraw, 2006). To determine whether Rab10 is required for this step, we measured the effect of Rab10 knockdown on GLUT4 redistribution using total internal reflection fluorescence (TIRF) microscopy (Gonzalez and McGraw, 2006), in which the total amount of HA-GLUT4-GFP within the evanescent excitation field is determined (based on GFP fluorescence). This assay is a measure of HA-GLUT4-GFP within \sim 250 nm (depth of the evanescent field) of the plasma membrane, and, unlike the HA-epitope exposure assay, which only measures HA-GLUT4-GFP inserted into the plasma membrane, the TIRF measurement includes HA-GLUT4-GFP in mobile vesicles and docked vesicles as well as in the plasma membrane. Thus, the TIRF assay can detect a redistribution of HA-GLUT4-GFP-containing vesicles to the plasma membrane even in the absence of vesicle fusion with the plasma membrane.

In insulin-stimulated Rab10 knockdown adipocytes, the fraction of HA-GLUT4-GFP within the evanescent field was reduced compared to control cells (Figure 7B). These data are in agreement with those in Figure 3B showing a reduction of GLUT4 in the plasma membrane by the HA-epitope exposure assay. The fraction of HA-GLUT4-GFP in the evanescent field was restored to the control level upon transient re-expression of Rab10 in the knockdown cells, showing that the effect is due to Rab10 knockdown. Knockdown of Rab10 did not affect the amount of HA-GLUT4-GFP in the evanescent field of basal cells (Figure 7C), consistent with the knockdown not affecting the behavior of GLUT4 in basal conditions (Figure 3B).

These data suggest that insulin-stimulated recruitment/docking of GLUT4-containing vesicles to the plasma membrane is attenuated by Rab10 knockdown. If the effect of Rab10 knockdown were limited to vesicle fusion, without an effect on recruitment/docking, then translocation would be arrested at the docked state and, in the simplest scenario, the amount of HA-GLUT4-GFP within the evanescent field would not be altered by Rab10 knockdown. We have recently shown that regulation of GLUT4 vesicle recruitment/docking is downstream of Akt (Gonzalez and McGraw, 2006). Thus, the data indicating that knockdown of Rab10 may effect insulin stimulation of recruitment/docking are consistent with Rab10 being a target of Akt regulation of AS160.

DISCUSSION

The data presented here strongly support the proposal that Rab10 is a component of the insulin-signaling transduction mechanism that regulates GLUT4 in the plasma membrane of adipocytes. Specifically, our findings support a model in which insulin-activated Akt phosphorylates and inhibits AS160 rab GAP activity, thereby shifting the Rab10 steady state to the GTP-bound form. This activation of Rab10 stimulates the exocytosis of GLUT4 and a coincident net increase of GLUT4 in the plasma membrane. Thus, along the Akt pathway, Rab10 is one of the most GLUT4 vesicle-proximal effectors of insulin action known. Although the role of AS160 as a mediator of insulin action is best documented in studies of cultured model adipocytes, recent studies have shown that AS160 is a likely mediator of insulin action in rodents and humans as well (Bruss et al., 2005; Deshmukh et al., 2006; Karlsson et al., 2006; Kramer et al., 2006a, 2006b; Treebak et al., 2006). Thus, Rab10 is likely an essential component of the cellular machinery that regulates whole-body glucose homeostasis.

Overexpression of the GTP-hydrolysis-defective Rab10-QL mutant induced an increase of GLUT4 in the plasma membrane of basal cells, indicating that the active form of Rab10 can overcome the inhibitory effects of AS160 on basal GLUT4 trafficking. This finding is consistent with AS160 being a negative regulator of Rab10 activation. Overexpression of wild-type Rab10 also resulted in an increase of GLUT4 in the plasma membrane of basal adipocytes, albeit to a smaller degree than Rab10-QL (Figure 1). Overexpression of wild-type Rab10 may also cause an increase in the GTP form, potentially by competition for AS160 GAP. Overexpression of neither wild-type Rab10 nor Rab10-QL had an effect on the amount of GLUT4 in the plasma membrane of insulin-stimulated cells. We interpret these data to indicate that the amount of Rab10-GTP is not normally limiting for GLUT4 translocation in insulin-stimulated adipocytes.

Knockdown of Rab10 altered the behavior of GLUT4 in insulin-stimulated adipocytes, but not basal adipocytes, providing evidence that Rab10 is specifically required for insulin-stimulated GLUT4 trafficking. In Rab10 knockdown adipocytes, insulin-stimulated translocation of

GLUT4 to the plasma membrane was reduced as a consequence of an attenuation of GLUT4 exocytosis (Figure 7A). Our data are consistent with Rab10 having a role in a pre-fusion GLUT4 vesicle recruitment/docking step (Figure 7B). Direct evidence supporting the proposal that Rab10 is a substrate of AS160 relevant for GLUT4 trafficking is provided by the finding that Rab10 knockdown partially blocks the increase of GLUT4 in the plasma membrane of adipocytes lacking AS160. We interpret these data to indicate that knockdown of AS160 results in a shift in the Rab10 steady state toward the GTP-bound form and a coincident increase of GLUT4 in the plasma membrane due to the stimulatory effect of Rab10-GTP on GLUT4 exocytosis. Knockdown of Rab10 in these cells reduces GLUT4 in the plasma membrane by reducing Rab10 and thereby removing inappropriate stimulation due to the loss of AS160.

The effects of Rab10 knockdown on insulin signaling to GLUT4 are not complete, accounting for only about one-third of the effect of insulin on GLUT4 translocation. The partial effect is not unexpected since there is considerable evidence that insulin signaling diverges to intersect GLUT4 trafficking at multiple points. For example, genetic or pharmacologic disruption of Akt inhibits only about two-thirds of the signaling to GLUT4 (Bae et al., 2003; Gonzalez and McGraw, 2006; Jiang et al., 2003). More relevant to the role of Rab10 in GLUT4 trafficking, knockdown of AS160 results in a 3-fold increase of GLUT4 in the plasma membrane, which indicates that, at a minimum, about one-third of the 10-fold translocation of GLUT4 to the plasma membrane is under AS160 regulation. Thus, the 30% decrease of GLUT4 in the plasma membrane in insulin-stimulated adipocytes that we observed for Rab10 knockdown is of the magnitude anticipated for disruption of an effector of insulin action downstream of AS160. Possibly these effects would be larger if the knockdowns of AS160 and Rab10 were complete.

Reversal of the increased plasma-membrane GLUT4 characteristic of basal AS160 knockdown adipocytes by knockdown of Rab10 provides compelling evidence that Rab10 is a bona fide target of AS160. The incomplete Rab10 knockdown reversal of the AS160 knockdown phenotype could be due to residual Rab10. AS160 knockdown cells may be particularly sensitive to Rab10 since, unlike control adipocytes, the AS160 knockdown adipocytes lack the negative regulation of Rab10 provided by AS160 GAP activity. Alternative explanations for the incomplete rescue of the AS160 phenotype are that AS160 regulates rab proteins in addition to Rab10 and/or that AS160 has functions beyond the GAP activity. Additional studies are required to fully document the downstream effectors of AS160 involved in insulin-stimulated GLUT4 translocation.

Rab10 and its closest relatives, Rab8 and Rab13, have roles in membrane trafficking in polarized mammalian cells (e.g., Ang et al., 2003; Morimoto et al., 2005; Pereira-Leal and Seabra, 2001). In MDCK cells, the site of Rab10 action has been localized to early/common endosomes of fully polarized cells and to the Golgi during early

times of differentiation, with Rab10 having a role in polarized intracellular trafficking in MDCK cell (Babbey et al., 2006; Schuck et al., 2007). In basal adipocytes, GLUT4 continually cycles between endosomes and GLUT4 vesicles (Bryant et al., 2002; Karylowski et al., 2004). Although we have not pinpointed the site of Rab10 regulation of GLUT4 trafficking, based on Rab10 function in MDCK cells and its localization in adipocytes, it is tempting to speculate that Rab10 may have a role in determining GLUT4 recycling kinetics by acting at the endosomal or intracellular sorting step.

There is evidence for insulin regulation of a number of steps of GLUT4 exocytosis, including the stimulation of the formation of GLUT4 transport vesicles (Xu and Kandror, 2002), the mobilization of a pool of GLUT4 vesicles (Bogan et al., 2003), the increased movement of GLUT4 vesicles (Bose et al., 2002; Semiz et al., 2003), and the increased docking of GLUT4 vesicles at the plasma membrane and/or increased fusion of GLUT4 vesicles with the plasma membrane (Bai et al., 2007; Koumanov et al., 2005; Lizunov et al., 2005; van Dam et al., 2005). Additional studies, including the identification of Rab10 effectors, are required to determine the mode of Rab10 action on GLUT4 trafficking.

While this work was in revision, another paper reported that overexpression of Rab8A and Rab14 overcame AS160-4P (constitutively active) inhibition of GLUT4 translocation in L6 muscle cells, whereas overexpression of Rab10 did not (Ishikura et al., 2007). These findings suggest that targets of AS160 that are involved in GLUT4 translocation might be cell-type specific, since we conclude from our present study that Rab10, and not Rab8A, is required for proper translocation of GLUT4 in 3T3-L1 adipocytes. We do not, however, rule out the possibility that one or more other rabs, such as Rab14, which is also a substrate for the GAP domain of AS160 *in vitro*, may also be involved in GLUT4 translocation of 3T3-L1 adipocytes. The incomplete effects of the rescue in AS160 knockdown cells by Rab10 knockdown might be the result of one or more other rabs' function. For example, the role of Rab10 in basolateral transport in MDCK cells was most apparent after additional inhibition of Rab8 (Schuck et al., 2007). To fully understand the role of AS160 in insulin-stimulated GLUT4 translocation, we need to find and further characterize other targets of AS160, including other rabs, as well as effectors of the rabs that are involved in the same pathway as AS160.

EXPERIMENTAL PROCEDURES

Antibodies

Fluorescent antibodies were purchased from Jackson Immunolabs, Inc. The concentration of HA.11 required to saturate the HA epitope of HA-GLUT4-GFP was determined for each preparation of antibody by measuring cell-associated HA.11 antibody after a 10 min pulse at 37°C (Karylowski et al., 2004). Typically, 50 µg/ml of HA.11 was a saturating concentration. An affinity-purified rabbit antibody prepared against a peptide unique to the carboxy-terminal regions of mouse Rab10 (amino acids 171–186) was used to detect endogenous Rab10 by immunoblotting. The antibodies against AS160 and IRAP

have been described previously (Keller et al., 1995; Sano et al., 2003). Other antibodies were from the following sources (name, catalog number, and source): actin, AAN01, Cytoskeleton; Akt1/2, H-136, and PPAR γ , sc-7196, Santa Cruz; Akt phospho-Thr308, 9721, Akt phospho-Ser473, 9271, and phospho-Akt substrate, 9614, Cell Signaling; Rab8, 610845, Rab4, 61088, and Rab11, 610656, BD Biosciences Pharmingen.

Cell Culture, Electroporation, and Plasmids

The methods for maintaining the cells, differentiation into adipocytes, and electroporation have been described previously (Zeigerer et al., 2002). The plasmids for HA-GLUT4-GFP and AS160 have been described previously (Kane et al., 2002; Lampson et al., 2001; Sano et al., 2003; Zeigerer et al., 2004). The plasmids for Rab10 and Rab10-QL are those described in Itoh et al. (2006); in each case, they direct the expression of the mouse protein with a FLAG tag at the amino terminus.

Preparation of shRNA Retroviral Constructs

The pSIREN RetroQ system from Clontech was used according to the manufacturer's instructions. AmphoPack packaging cells were transfected with the retroviral cDNA (Clontech), and culture medium from the packaging cells, harvested between 24 and 48 hr posttransfection, was used to infect 3T3-L1 cells (Wertheim et al., 2004). The infected cells were selected for growth in puromycin, the surviving cells were differentiated, and the behavior of GLUT4 was characterized. Knockdown cells were not carried for more than two passages. Over the course of this study, multiple batches of both Rab10 KD(87) and KD(251) cells were prepared, and we did not find any significant variation in the behavior of GLUT4 among cells derived from different infections. The control retrovirus expressing a shRNA not targeting any mouse gene has been described previously (Eguez et al., 2005).

Kinetic Studies

All live cell incubations were performed in serum-free DMEM with 20 mM sodium bicarbonate, 20 mM HEPES (pH 7.2) (SF-DMEM) at 37°C in 5% CO $_2$. In all experiments, adipocytes were preincubated in SF-DMEM medium for at least 180 min at 37°C in 5% CO $_2$ /air (basal conditions). For indirect immunofluorescence, cells were fixed with 3.7% formaldehyde; incubated with primary antibody in 150 mM NaCl, 20 mM HEPES, 1 mM CaCl $_2$, 5 mM KCl, 1 mM MgCl $_2$ (pH 7.2) (med 1) with 1% calf serum for 60 min at 37°C, washed extensively with med 1, and incubated with fluorescent secondary antibody in med 1 with 1% calf serum. For indirect immunofluorescence of permeabilized cells, med 1 with 250 µM saponin was used for incubations and washes.

The methods for measuring the surface-to-total distribution of HA-GLUT4-GFP, IRAP-TR, and TR have been described previously (Karylowski et al., 2004; Lampson et al., 2001; Zeigerer et al., 2002, 2004). The methods for measuring the efflux kinetics of HA-GLUT4-GFP have been described previously in detail (Karylowski et al., 2004; Zeigerer et al., 2004). Briefly, GLUT4 exocytosis was measured by incubating adipocytes in SF-DMEM containing a saturating concentration of HA.11 antibody at 37°C in 5% CO $_2$ /air. The amount of cell-associated HA.11 was determined by indirect immunofluorescence of saponin-permeabilized cells using a Cy3-labeled goat anti-mouse-IgG.

Fluorescence Quantification

All epifluorescence images were collected on a DMIRB inverted microscope at room temperature using a 40 \times 1.25 numerical aperture oil-immersion objective (Leica Microsystems) and a cooled CCD 12-bit camera (Princeton Instruments). Exposure times for each fluorescence channel were chosen such that >95% of the image pixel intensities were below camera saturation. Exposure times were kept constant within each experiment. Image quantification was performed as described previously (Dunn et al., 1994; Lampson et al., 2000) using MetaMorph image processing software (Universal Imaging). Images

were background corrected using nonexpressing cells chosen from within the same fields as the expressing cells.

Total Internal Reflection Fluorescence Microscopy

To measure GLUT4 translocation, adipocytes were incubated in serum-free DMEM medium for 3 hr at 37°C in 5% CO₂/air. Cells were fixed in 3.7% formaldehyde, and the insulin-induced GLUT4 redistribution was determined using a TIRF microscope (described previously; Moskowitz et al., 2003). A 60× 1.45 numerical aperture oil-immersion objective (Olympus America) was used to perform "prismless" TIRF. The evanescent field decay length was ~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 the microscope's epifluorescence mode. Both epifluorescence and TIRF images of cells were acquired. The GFP fluorescence in the TIRF mode was divided by the GFP epifluorescence intensity, normalizing the TIRF fluorescence for the total HA-GLUT4-GFP expressed per cell. All images were corrected for background fluorescence measured in cells that did not express HA-GLUT4-GFP.

Generation of Diced siRNA Pools

Pools of diced siRNAs targeting firefly luciferase and mouse Rab10 were generated using methods described previously (Myers et al., 2003). The entire 603 bp sequence of mouse Rab10 (NCBI accession number [NM_01676](#)) was used as the template for in vitro transcription in in vitro dicing. Diced siRNA pools were electroporated into 3T3-L1 adipocytes at a concentration of 30 nM.

Supplemental Data

Supplemental Data include one figure and Supplemental References and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/5/4/293/DC1>.

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