Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein

Lorena Eguez,[1] Adrian Lee,[2] Jose A. Chavez,[3] Cristinel P. Miinea,[3] Susan Kane,[3] Gustav E. Lienhard,[3] and Timothy E. McGraw[1,2,*]

[1]Department of Biochemistry, Weill-Cornell Medical School, New York, New York 10021
[2]Training Program In Chemical Biology, Weill-Cornell Medical School, New York, New York 10021
[3]Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755-3844
*Correspondence: temcgraw@med.cornell.edu

Summary

Insulin controls glucose flux into muscle and fat by regulating the trafficking of GLUT4 between the interior and surface of cells. Here, we show that the AS160 Rab GTPase activating protein (GAP) is a negative regulator of basal GLUT4 exocytosis. AS160 knockdown resulted in a partial redistribution of GLUT4 from intracellular compartments to the plasma membrane, a concomitant increase in basal glucose uptake, and a 3-fold increase in basal GLUT4 exocytosis. Reexpression of wild-type AS160 restored normal GLUT4 behavior to the knockdown adipocytes, whereas reexpression of a GAP domain mutant did not revert the phenotype, providing the first direct evidence that AS160 GAP activity is required for basal GLUT4 retention. AS160 is the first protein identified that is specially required for basal GLUT4 retention. Our findings that AS160 knockdown only partially releases basal GLUT4 retention provides evidence that insulin signals to GLUT4 exocytosis by both AS160-dependent and -independent mechanisms.

Introduction

One acute effect of insulin is to stimulate the recruitment of the GLUT4 glucose transporter from intracellular compartments to the plasma membrane (PM) of fat and muscle cells (Watson et al., 2004). Most evidence supports a model in which the amount of GLUT4 in the PM is dynamic, being determined by the rates of exocytosis and endocytosis (Holman and Cushman, 1994; Jhun et al., 1992; Karylowski et al., 2004; Li et al., 2001; Rudich and Klip, 2003; Yang and Holman, 1993; Yeh et al., 1995). In basal adipocytes, GLUT4 is slowly exocytosed and rapidly internalized and upon insulin stimulation, exocytosis is accelerated and endocytosis is inhibited, resulting in a net increase of GLUT4 on the PM.

Insulin receptor activation of the Akt/PKB serine/threonine kinase is necessary for redistribution of GLUT4 to the cell surface, although there is debate as to whether it is sufficient (Bae et al., 2003; Cho et al., 2001; Jiang et al., 2003; Kanzaki et al., 2004). Recently, a substrate of Akt has been identified, AS160, that functions in insulin signaling to GLUT4 movement (Kane et al., 2002; Sano et al., 2003). AS160, also known as TBC1D4, has homology with members of the Rab GTPase activating protein (GAP) family. Rab proteins are GTPases involved in the regulation of many membrane trafficking processes (Deneka et al., 2003; Spang, 2004; Zerial and McBride, 2001). The GTP bound form of Rab proteins is considered the "active state" and the GDP bound form the "inactive state." Conversion from one nucleotide bound form to the other is tightly regulated by accessory factors, including GAP proteins, which stimulate GTP hydrolysis and drive the equilibrium toward the GDP form, and guanine nucleotide exchange factors, which stimulate the exchange of GTP for GDP and promote activation of Rab proteins. AS160 constructs in which Akt phosphorylation sites have been mutated to alanines inhibit insulin-stimulated redistribution of GLUT4 to the PM (Sano et al., 2003; Zeigerer et al., 2004). It was proposed that AS160 is a negative regulator of GLUT4 trafficking in basal conditions, with its GAP activity functioning to maintain a Rab protein(s) that regulates GLUT4 redistribution to the PM in the "inactive" GDP bound form, and that insulin stimulation results in an inhibition of AS160 GAP and a concomitant accumulation of the active GTP bound Rab (Sano et al., 2003).

Here, we show that knockdown of AS160 in adipocytes results in a partial redistribution of GLUT4 to the PM in basal conditions. The decreased basal intracellular retention of GLUT4 is correlated with an increased GLUT4 exocytosis in unstimulated AS160 knockdown adipocytes. Insulin induces a further increase of GLUT4 in the PM of the knockdown adipocytes by stimulating GLUT4 exocytosis. Transient expression of wild-type AS160, but not a GAP domain mutant of AS160, rescues the AS160 knockdown phenotype, providing the first direct evidence that AS160 GAP activity is required for basal GLUT4 retention. The effects of AS160 knockdown are specific for insulin-regulated trafficking pathway, as the trafficking of IRAP is affected by AS160 knockdown but the behavior of the transferrin receptor is not. In studies of the behavior of GLUT4 during the transition from basal to insulin-stimulated states, we show that about 40% of GLUT4 is transiently recruited to the PM of adipocytes expressing a dominant-inhibitory constitutively active GAP AS160 mutant. This biphasic behavior indicates that Akt phosphorylation of AS160 is required for maintaining redistribution of GLUT4 to the PM, but not for all of the initial recruitment. The results of this study demonstrate AS160 is specifically required for GLUT4 retention and they suggest that factors in addition to AS160 are required for the full intracellular retention in basal conditions.

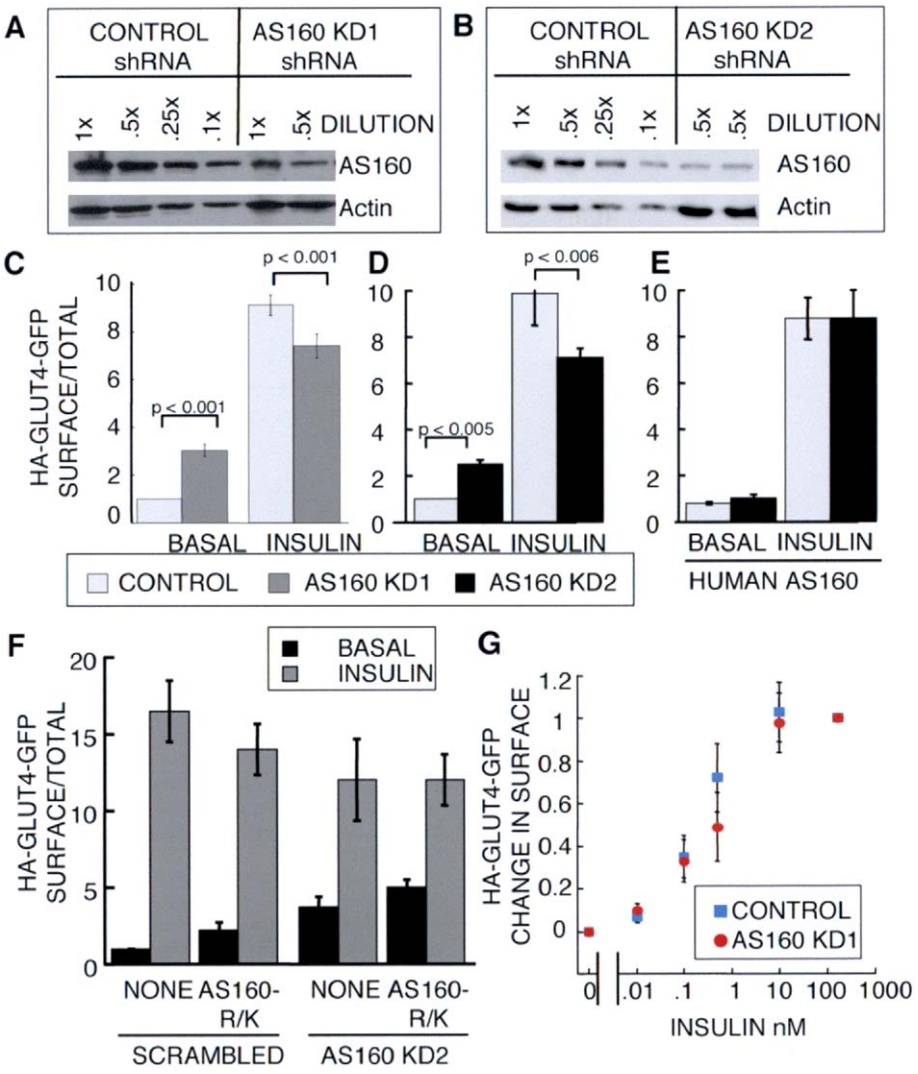

Figure 1. shRNA knockdown of mouse AS160 affects expression of GLUT4 on plasma membrane, is rescued by expression of human AS160, and does not change the insulin-dose response

A and B) Western blotting of extracts from adipocytes on day 5 postdifferentiation expressing a control shRNA that does not target any mouse gene or shRNA AS160 KD1 or shRNA AS160 KD2. Extracts were prepared from the same number of cells, and dilutions of the extracts, noted at top of the gel, were blotted for AS160 and for actin. The actin levels serve as gel loading controls. In (**B**), the two KD2 extracts were from adipocytes prepared from different infections with the KD2 shRNA.

C–F) Surface-to-total distributions of HA-GLUT4-GFP in basal and insulin conditions. The surface-to-total value is the ratio of the HA.11 on surface (measured in indirect immunofluorescence) to the GFP in individual cells. This ratio is proportional to the fraction of GLUT4 on the cell surface. Insulin-stimulated cells were incubated with 170 nM insulin for 30 min.

C) The average ±SEM of ten independent paired experiments comparing the control shRNA to shRNA AS160 KD1 are shown. The paired, two-tailed t test probabilities (p) are shown.

D) The data are the average of five experiments ±SEM comparing control to AS160 KD2.

E) The average ±SEM of five experiments from control and AS160 KD2 adipocytes coelectroporated with HA-GLUT4-GFP and wild-type human AS160 are shown.

F) The average ±SD of three experiments from control and AS160 KD2 adipocytes electroporated with HA-GLUT4-GFP alone (none) or coelectroporated with HA-GLUT4-GFP and the human AS160 R/K mutant are shown.

G) The amount of HA-GLUT4-GFP on the surface of control and AS160 KD1 adipocytes as a function of 30 min incubation in various concentrations of insulin. To compare the response of the control and AS160 KD1 cells, which have different amounts of HA-GLUT4-GFP on the surface in basal and optimal insulin, the fractional change in surface HA-GLUT4-GFP is plotted, which is the difference in HA-GLUT4-GFP on the surface at each insulin concentration minus the amount on the surface in basal, divided by the change in surface HA-GLUT4-GFP measured between 0 and 170 nM insulin. The data are the average of three experiments ±SD.

Results

Reduction of AS160 by small-inhibitor RNA increases GLUT4 on the surface of basal adipocytes

To further assess the role of AS160 in insulin-regulation of GLUT4 trafficking, we knocked down AS160 using retroviral constructs containing short-hairpin RNA (shRNA) targeting AS160 sequences. We also created a retrovirus containing shRNA sequences that do not target any known gene in the mouse genome as a control (Mitra et al., 2004). The replication-defective retroviruses were used to infect 3T3-L1 cells, which were then selected for growth in puromycin. The surviving cells were differentiated and the amount of AS160 examined by Western blotting (Figures 1A and 1B). We chose to study two of the shRNA constructs that resulted in a pronounced reduction of AS160. Both of these AS160 shRNA constructs, AS160 Knock Down 1 (AS160 KD1): GAGCAAGCCTTTGAAATGC and AS160 Knock Down 2 (AS160 KD2: GACTTAACTCATCCAA CGA, reduced AS160 by 70% to 90%.

A GLUT4 reporter containing an exofacial HA epitope and GFP fused to the carboxyl cytoplasmic domain, HA-GLUT4-GFP, was used to characterize the behavior of GLUT4 in the AS160 KD adipocytes (Zeigerer et al., 2002). Knockdown of AS160 resulted in a 3-fold increase of GLUT4 in the PM of basal adipocytes and an approximate 20% reduction of GLUT4 on the surface of insulin-stimulated adipocytes (Figures 1C and 1D). The increase in HA-GLUT4-GFP in the PM was measured by an increase in anti-HA epitope antibody (HA.11) staining on the cell surface, which measures HA-GLUT4-GFP that has been inserted into the PM. The increased surface expression of GLUT4 in basal AS160 KD cells demonstrates a role for AS160 in basal intracellular retention of GLUT4, establishing that AS160 is a negative regulator of GLUT4 movement to the

PM. The reduction of GLUT4 on the PM of insulin-stimulated knockdown cells provides evidence that AS160 has a role in determining the amount of GLUT4 in the PM in the stimulated conditions as well. Knockdown of AS160 using the two different AS160 shRNA targeting sequences resulted in similar phenotypes, indicating that the change in GLUT4 behavior was due to the specific knockdown of AS160.

The sequences targeted by AS160 KD2 are not identical between mouse and human (mouse, GACTTAACTCATCCAA CGA, and human, GACCTAAACTGCAACCCTA). Identity of sequence is required for efficient RNAi gene silencing. To confirm that the change in GLUT4 behavior was due to a knockdown in AS160, we determined whether human AS160 could rescue the effects of KD2. In AS160 KD2 adipocytes transiently expressing human AS160, the surface-to-total distribution of HA-GLUT4-GFP was indistinguishable from that in control cells, in both basal and insulin-stimulated conditions (Figure 1E). Thus, the alteration in GLUT4 distribution in the AS160 KD cells is due to a reduction of AS160 and not due to a change in expression of some other protein. The sequences in KD1 are identical in both mouse and human AS160, and therefore it was not possible to test for rescue by transfection with human AS160.

A functional AS160 GAP domain is required for basal GLUT4 retention

The ability to rescue the AS160 KD phenotype of KD2 cells by transient transfection of human AS160 provides a means of testing whether the GAP domain of AS160 is required for basal retention of GLUT4 (Sano et al., 2003). Transfection of AS160 KD2 cells with a previously described AS160 R/K mutant, in which the critical arginine of the GAP domain has been mutated to lysine, thereby inactivating the GAP activity (Miinea et al., 2005; Sano et al., 2003), does not restore basal GLUT4 intracellular retention (Figure 1F). These data provide the first direct evidence that a functional AS160 GAP domain is required for basal retention of GLUT4.

AS160 knockdown does not affect insulin signal transduction

The insulin dose-response in AS160 KD adipocytes was unchanged compared to control cells, which document that the phenotype of the AS160 KD cells results from a change in trafficking of GLUT4 rather than a change in sensitivity to insulin (Figure 1G). Furthermore, HA-GLUT4-GFP on the surface of AS160 KD adipocytes rapidly increased after addition of insulin, reaching a steady-state surface level within 10 min (see Figure S1 in the Supplemental Data available with this article online). The rise to a new stable plateau was similar to the change in surface GLUT4 in control cells, demonstrating that the reduced GLUT4 translocation in the AS160 KD cells is not due to a delay in redistribution of GLUT4 to the cell surface.

Insulin-stimulated redistribution of GLUT4 to the PM requires activation of phosphoinositide 3 kinase (PI 3 kinase). 100 nM wortmannin, a PI 3 kinase inhibitor, inhibited insulin-stimulated translocation of HA-GLUT4-GFP to the PM of AS160 KD cells, without affecting the amount of HA-GLUT4-GFP in the PM of basal AS160 KD adipocytes (Figure S1). Thus, insulin recruits GLUT4 to the PM of AS160 KD adipocytes by a PI 3 kinase-dependent mechanism, and the increase of GLUT4 in the PM of basal AS160 KD adipocytes is not due to an inappropriate activation of PI 3 kinase.

The amount of AS160 expressed is not linearly correlated with GLUT4 retention

Translocation of GLUT4 is measured in single cells using the HA-GLUT4-GFP reporter (Lampson et al., 2001; Zeigerer et al., 2002). It was of interest to determine, at an individual cell level, whether there is a linear (or graded) correlation between the amount of AS160 expressed and the degree of GLUT4 retention. In control adipocytes, AS160 was in a punctate distribution, with some concentration in the perinuclear region (Figure 2A). There was only a partial overlap between HA-GLUT4-GFP and AS160, most prominently in the perinuclear region. The reduction in AS160 was readily apparent in the KD cells. The HA-GLUT4-GFP (GFP fluorescence) pattern was not greatly altered in the AS160 KD cells, although there was an increase in diffuse GFP, probably reflecting HA-GLUT4-GFP in the PM. The 3-fold increase in HA-GLUT4-GFP in the PM was evident in the increased HA.11 staining of AS160 KD adipocytes.

In control cells, there was a 3-fold range of expression of AS160 among individual cells; however, the degree of GLUT4 retention was constant over this range and there was no correlation between AS160 expression and the insulin-stimulated redistribution of GLUT4 to the PM (Figure 2B). The majority of the AS160 KD cells expressed AS160 at a level below the range observed in controls cells, and the distribution of GLUT4 in basal AS160 KD cells was shifted toward the cell surface. There was no linear or graded correlation between the amount of AS160 expressed in the KD cells and the surface-to-total distribution of GLUT4. Rather, it appears as if a certain amount of AS160 is required for proper basal GLUT4 retention and once the amount drops below this threshold there is a partial redistribution of GLUT4 to the PM. The GLUT4 surface-to-total distribution in insulin-stimulated AS160 KD cells was not linearly correlated with the amount of AS160 expressed per cell.

AS160 knockdown elevates basal glucose transport

In both control and AS160 KD basal adipocytes, endogenous GLUT4 was localized to the perinuclear region and distributed throughout the cytoplasm in small punctate structures (Figure 2C). In some of the KD adipocytes, the increase in surface GLUT4 was apparent, but this was not the case for all AS160 KD cells. The expression of endogenous GLUT4 in AS160 KD cells provides evidence that the knockdown of AS160 did not grossly inhibit differentiation (also see below). Staining of GLUT4 in insulin-stimulated control and AS160 KD adipocytes did not reveal any significant differences, with the redistribution of GLUT4 into the PM apparent in both cases (Figure 2C).

In qualitative agreement with GLUT4 distribution measured by HA-GLUT4-GFP, basal ^{3}H-2-deoxy-glucose uptake into AS160 KD adipocytes was about 2-fold higher than in adipocytes expressing the control shRNA (Figure 2D). Insulin-stimulated glucose uptake into AS160 KD adipocytes was also reduced by a small amount, consistent with the small reduction in HA-GLUT4-GFP on the surface of insulin-stimulated AS160 KD cells.

The quantitative differences in GLUT4 behavior between control and AS160 KD cells measured by hexose uptake are smaller than measured by HA-GLUT4-GFP (Figures 1 and 2D). This is not surprising since hexose uptake is not a direct mea-

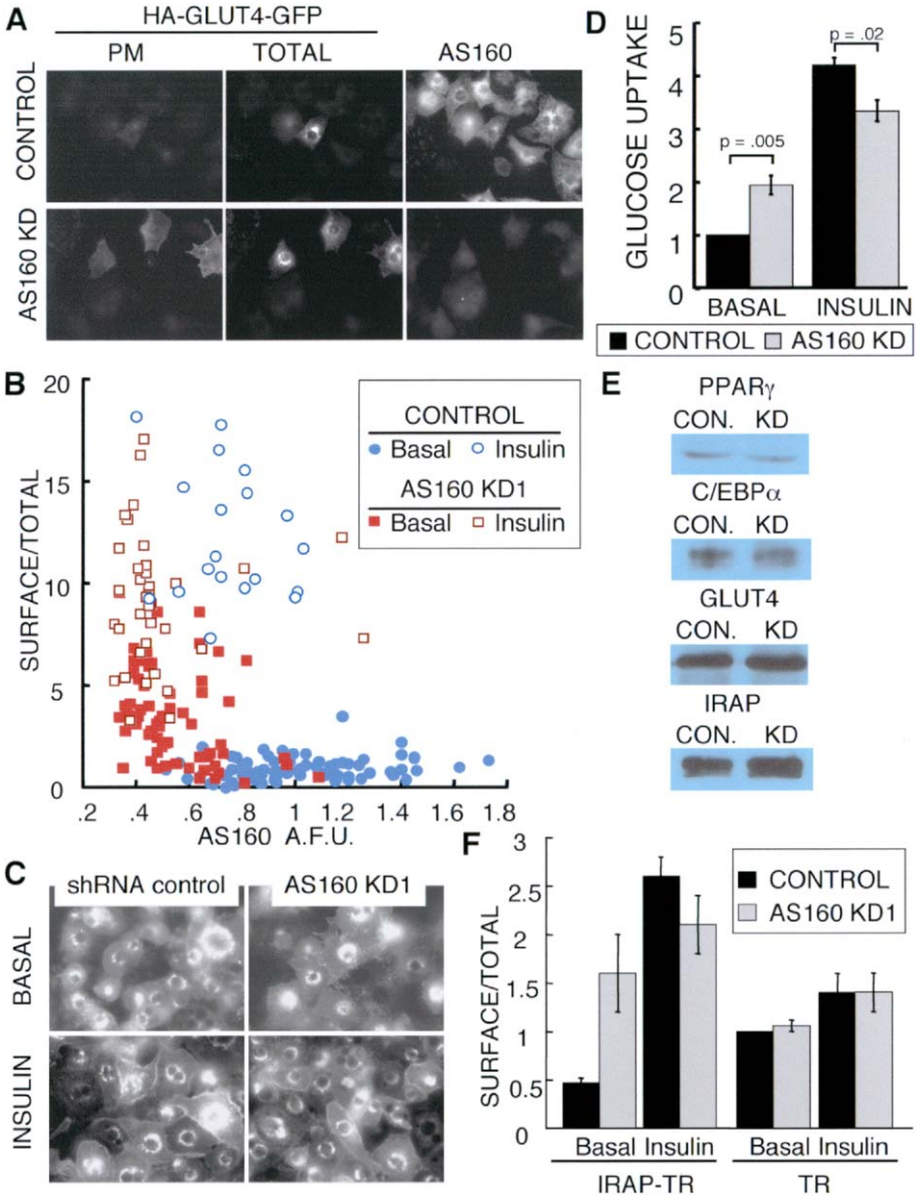

Figure 2. The effect of AS160 KD on GLUT4 distribution examined in individual cells, on hexose uptake, on differentiation, and on the behavior of IRAP and TR

A) The surface-to-total HA-GLUT4-GFP distributions and the amount of AS160 expressed were determined in unstimulated individual control and AS160 KD1 cells. Surface HA-GLUT4-GFP was quantified by indirect immunofluorescence of HA.11 of fixed cells. Following staining for surface HA.11, the cells were permeabilized and AS160 quantified by indirect immunofluorescence. The images were collected with a 40× objective.

B) Each symbol is the surface-to-total HA-GLUT4-GFP distribution in an individual cell. The data for the control shRNA basal adipocytes and the basal AS160 KD1 are pooled from three experiments; the data for the insulin-stimulated AS160 KD1 are from two experiments, and the data for control shRNA insulin-stimulated adipocytes are from a single experiment. In each experiment, the surface-to-total values of the individual cells were normalized to the average surface-to-total value of the control basal cells in that experiment, and the amount of AS160 per cell, detected by indirect immunofluorescence, was normalized to the average AS160 in control basal cells in that experiment. The AS160 immunofluorescence values shown were not corrected for background. The AS160 fluorescence is in arbitrary fluorescence units (AFU) with 1 being the average value in control cells. Surface-to total values from 83 basal and 19 insulin-stimulated shRNA control adipocytes and 69 basal and 37 insulin-stimulated KD1 adipocytes are plotted.

C) Images of endogenous GLUT4 in basal and insulin-stimulated control and AS160 KD1 adipocytes 5 days postdifferentiation determined by indirect immunofluorescence. Images were collected using a 40× objective.

D) ^{3}H-2-deoxy-glucose uptake in adipocytes expressing control shRNA or AS160 KD1 shRNA. The average ±SD of four experiments measuring uptake in day 10 adipocytes are shown. In each experiment, the uptake of ^{3}H-2-deoxy-glucose was normalized to the uptake in basal control adipocytes. Paired, two-tailed t test probabilities (p) are shown.

E) Western blot analysis of protein expression in day 5 shRNA control and AS160 KD2 adipocytes. Extracts of each cell type were prepared from the same number of adipocytes and the same volume of extract was run per lane of the gel.

F) The average ±SD of the surface-to-total distribution of the IRAP-TR chimera and the human TR determined in three experiments are shown. The data from the individual experiments are normalized to the surface-to-total value for the TR in basal adipocytes expressing the control shRNA construct.

sure of GLUT4 in the PM, as GLUT1 in the PM also contributes to both basal and insulin-stimulated ^{3}H-2-deoxy-glucose uptake. There was no difference in the amount of GLUT1 expressed in AS160 KD cells and control adipocytes (not shown) and therefore the changes in ^{3}H-2-deoxy-glucose uptake in AS160 KD adipocytes are likely due to an effect of AS160 on the amount of endogenous GLUT4 in the PM in basal and insulin-stimulated conditions, with the magnitude of the changes measured by hexose uptake blunted by the contribution of GLUT1.

AS160 knockdown adipocytes differentiated normally

The AS160 KD adipocytes are morphologically indistinguishable from control adipocytes, with both accumulating significant lipids by day 5 of differentiation (Figure S2). Both PPARγ and C/EBPα, two transcription factors critical for adipocyte differentiation (Rosen, 2002), are properly expressed in the AS160 KD cells (Figure 2E). Two additional markers of differentiation are GLUT4 and IRAP, both of which are properly expressed in the AS160 KD cells (Figure 2E). These data argue that the defects in GLUT4 trafficking in AS160 KD cells are not due to a defect in differentiation of the AS160 KD adipocytes. Furthermore, the fact that transient reexpression of AS160 KD adipocytes reverts the GLUT4 trafficking to wild-type (Figure 1E) also provides evidence that the KD phenotype is due to loss of AS160 and not due to altered differentiation.

AS160 KD affects IRAP but not transferrin-receptor distribution

IRAP, a transmembrane aminopeptidase, is the only other membrane protein known to traffic with GLUT4 by the insulin-

regulated recycling pathway (Keller et al., 2002; Ross et al., 1997). As a reporter for IRAP behavior, we used a chimera in which the cytoplasmic domain of IRAP is substituted for that of the transferrin receptor (Johnson et al., 1998; Subtil et al., 2000; Zeigerer et al., 2002). AS160 knockdown induced a redistribution of IRAP-TR to the PM of basal adipocytes as well as a small reduction in the amount of IRAP-TR in the PM of stimulated cells (Figure 2F). The insulin-stimulated fold translocation of IRAP-TR to the PM of AS160 KD adipocytes was smaller than that of HA-GLUT4-GFP (Figures 1C and 1D to Figure 2F). We do not know the reason for this small difference. Regardless, the change in behavior of IRAP-TR is consistent with AS160 knockdown affecting the insulin-regulated pathway and not just GLUT4.

The transferrin receptor (TR) is a reporter of general endocytic trafficking. The distribution of the TR between the interior and cell surface is only slightly affected by insulin (increased by 2-fold or less; Tanner and Lienhard, 1987; Zeigerer et al., 2002). AS160 knockdown had no significant effect on the surface-to-total distribution of the TR in basal or insulin stimulated conditions, indicating that reduction of AS160 does not perturb general endocytic trafficking (Figure 2F).

AS160 knockdown increases basal GLUT4 exocytosis by 3-fold

We next compared the recycling kinetics of HA-GLUT4-GFP in control and AS160 KD adipocytes (Figure 3A). Exocytosis is measured by incubating cells in medium containing anti-HA epitope antibody HA.11. Any HA-GLUT4-GFP that appears on the surface during the incubation is bound by HA.11 and remains bound regardless of whether it remains on the surface or is internalized (Karylowski et al., 2004; Zeigerer et al., 2002, 2004). Total cell-associated HA.11 increases with time of incubation until all HA-GLUT4-GFP has cycled to the PM, with the rise to this plateau level determined by the exocytosis rate constant. Cell-associated HA.11 reached a plateau by 200 min in AS160 KD adipocytes, whereas a plateau was not reached until about 500 min in control adipocytes. The exocytosis rate constants calculated from these data demonstrated that GLUT4 was recycled to the cell surface about 3-fold faster in basal AS160 KD adipocytes than in control cells, directly establishing a role for AS160 in the regulation of basal GLUT4 exocytosis (Figure 3B).

The same method was used to examine the exocytosis of GLUT4 in insulin-stimulated adipocytes. In this case, the cells were incubated with insulin for 30 min and then incubated in medium with insulin and HA.11 antibody. Insulin stimulated a 3- to 4-fold increase in GLUT4 exocytosis in AS160 KD adipocytes (Figures 3C and 3D). Exocytosis of GLUT4 in the AS160 KD cells was slightly slower than in control cells, although this trend did not reach statistical significance.

AS160 knockdown affects the distribution of GLUT4 between endosomes and specialized GLUT4/IRAP compartment

We next used an HRP ablation method to examine the distribution of HA-GLUT4-GFP between TR-containing endosomes and the GLUT4/IRAP specialized compartment (Lampson et al., 2001; Martin et al., 1996; Zeigerer et al., 2002). HRP-Tf is delivered to endosomes by uptake via the TR and to endosomes and the GLUT4/IRAP specialized compartment by uptake via IRAP-TR (Karylowski et al., 2004; Zeigerer et al., 2002). HA.11 epitopes in the same compartments as the internalized HRP-Tf are ablated by the HRP-catalyzed polymerization of diaminobenzidene. In control adipocytes, HRP-Tf delivered to cells by IRAP-TR ablated nearly all the internalized HA.11, indicating extensive colocalization of IRAP-TR and HA-GLUT4-GFP, whereas HRP-Tf delivered by TR ablated about half of the HA.11, indicating that only half of the HA-GLUT4-GFP in basal adipocytes was in TR-containing endosomes (Figures 3E and 3F). In basal AS160 KD cells, there was a significant increase in ablation of HA.11 with HRP-Tf internalized by TR and no change in the ablation by HRP-Tf internalized by IRAP-TR (Figure 3E). In basal AS160 KD adipocytes, about 70% of intracellular GLUT4 was in TR-containing endosomes, compared to about 50% in control adipocytes (Figure 3F). Thus, AS160 knockdown resulted in a shift in the intracellular distribution of GLUT4 from the GLUT4/IRAP specialized compartment to endosomes.

Insulin transiently recruits GLUT4 to the PM in cells expressing a dominant-interfering AS160

We have previously shown that expression of the AS160 mutated in four of the six Akt phosphorylation sites, which we refer to here as dominant-interfering AS160 (DI-AS160, which has been referred to as AS160-4P), abrogates the steady-state accelerated exocytosis kinetics of GLUT4 in the presence of insulin (Sano et al., 2003; Zeigerer et al., 2004). To examine the effect of the DI-AS160 on the transition of GLUT4 from basal to insulin-stimulated conditions, we compared the amount of HA-GLUT4-GFP on the surface of cells as a function of time in insulin (Figure 4A). In control adipocytes, the amount of GLUT4 on the surface rapidly increased to a new plateau level within 10 min of insulin treatment, and this time was not affected by overexpression of wild-type AS160. In cells expressing DI-AS160, there was a rise in surface HA-GLUT4-GFP, peaking at about five times the basal level within 5 min of insulin treatment (Figure 4B). However, unlike the case in control cells, this increase in surface HA-GLUT4-GFP was only transiently maintained, with surface GLUT4 decaying by 15 min to a plateau about 3-fold above basal. The reduced steady-state redistribution of GLUT4 to the PM of cells expressing DI-AS160, which was about 30% of the control translocation, was in agreement with the results of previous studies (Sano et al., 2003; Zeigerer et al., 2004). The transient rise in surface GLUT4 indicates that in cells expressing DI-AS160, insulin can initially recruit GLUT4 to the surface but this increase cannot be fully maintained.

To more closely examine the behavior of GLUT4 during the transition from basal to insulin stimulated conditions, we examined how much of the GLUT4 is rapidly recruited to the cell surface DI-AS160 adipocytes. HA.11 and insulin were simultaneously added to cells, so that HA-GLUT4-GFP rapidly recruited to the surface would be bound by HA.11. In control cells, total cell-associated HA.11 leveled off at about 30 to 40 min, and there was no significant increase in cell-associated HA.11 when the incubation was continued for 480 min (Figure 4C). These data show that the insulin-recruitable intracellular pool of HA-GLUT4-GFP moved to the PM within 40 min of insulin addition, consistent with previous observations (Govers et al., 2004; Karylowski et al., 2004). In DI-AS160 adipocytes, the increase in total cell-associated HA.11 was biphasic. Approximately 40% of intracellular GLUT4 was labeled within 40 min of insulin addition (Figure 4C). Upon prolonged incubation,

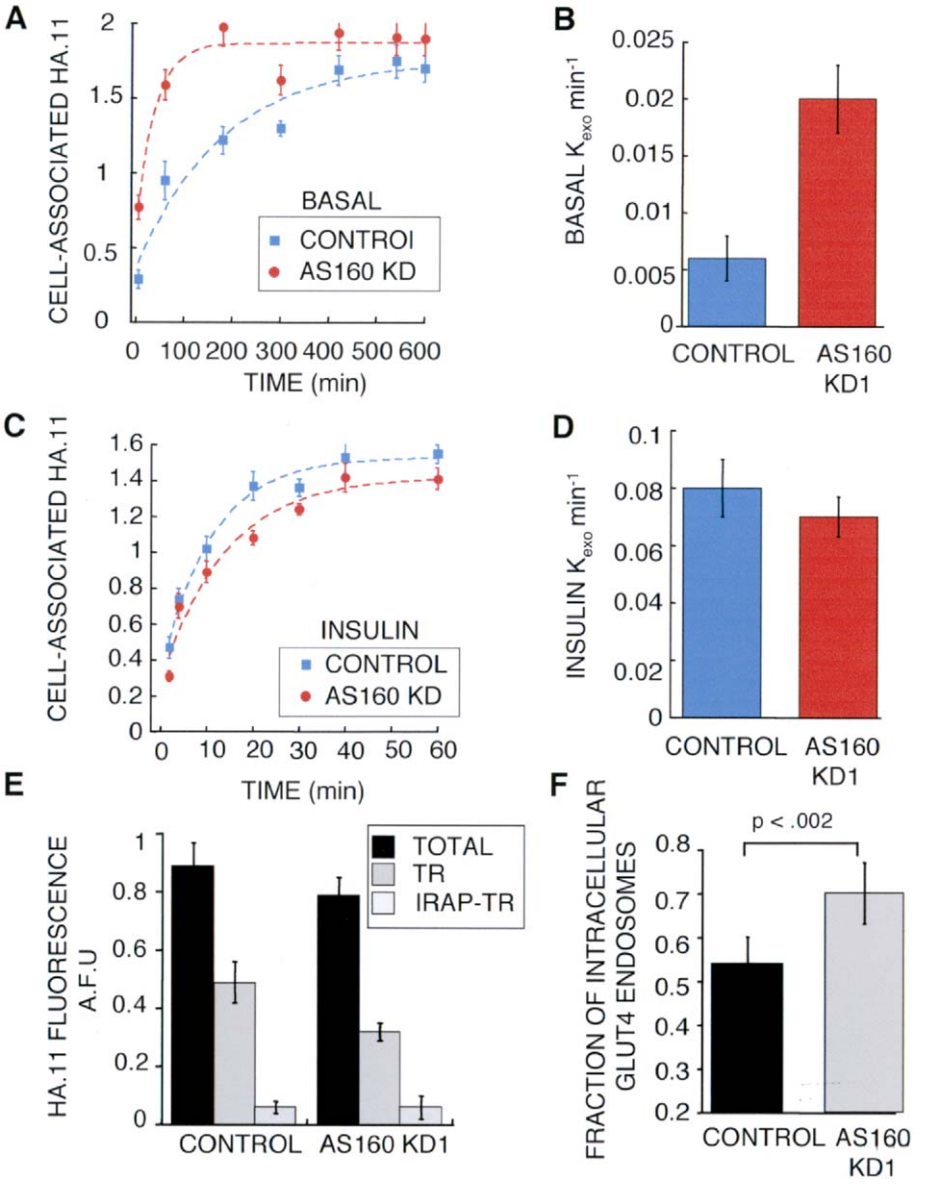

Figure 3. HA-GLUT4-GFP exocytosis kinetics and distribution among intracellular compartments

A) A representative experiment of basal GLUT4 exocytosis kinetics. The data are the average ±SEM of at least 20 cells per time point. Cell-associated HA.11 was calculated as the ratio of HA.11, measured by indirect immunofluorescence with a Cy3-labeled goat anti-mouse-IgG. The GFP fluorescence was used as a measure of total HA-GLUT4-GFP expressed per cell. All points were collected using the same Cy3 and GFP exposure times. The lines are a fit of the data to $(Cy3/GFP)_t = (HA\text{-}GLUT4\text{-}GFP)_{plateau} - [(HA\text{-}GLUT4\text{-}GFP)_{intracellular}*(e^{-kexo*t})]$; where k_{exo} is the exocytosis rate constant, t is time of incubation, $(Cy3/GFP)_t$ is cell-associated HA.11 at time t, $(HA\text{-}GLUT4\text{-}GFP)_{plateau}$ is the Cy3-to-GFP plateau level, and $(HA\text{-}GLUT4\text{-}GFP)_{intracellular}$ is the amount of HA-GLUT4-GFP intracellular at time 0. In this experiment, the k_{exo} measured were 0.006 min^{-1} and 0.025 min^{-1} for the control and AS160 KD, respectively.

B) The average basal k_{exo} measured in four independent experiments ±SD.

C) A representative experiment of steady-state insulin-stimulated GLUT4 exocytosis kinetics. Cells were prestimulated with 170 nM insulin for 30 min and the time course of HA.11 uptake in the continued presence of insulin was measured. The data are the average ±SEM of at least 20 cells per time point. The lines are a fit of the data to the equation as discussed for (**A**). In this experiment, the insulin-stimulated k_{exo} measured were 0.09 min^{-1} and 0.07 min^{-1} for the control and AS160 KD, respectively.

D) The average insulin-stimulated k_{exo} measured in three independent experiments ±SD.

E) Distribution of GLUT4 between endosomes and the specialized GLUT4/IRAP compartment is altered by AS160 knockdown. Colocalization of HA-GLUT4-GFP with TR and IRAP-TR determined by HRP ablation. Data from a representative experiment are shown. The values are the amount of HA.11 detected by immunofluorescence of permeabilized cells, normalized to the GFP fluorescence per cell. The data are the average of values from at least 20 cells ±SEM. Cells cotransfected with HA-GLUT4-GFP and TR, or HA-GLUT4-GFP and IRAP-TR, were incubated with HA.11 antibody and Tf-HRP for 240 min. The no-ablation control cells were incubated in HA.11 antibody but no Tf-HRP. Tf-HRP in intracellular compartments converts DAB in the presence of H_2O_2 into a polymer that ablates epitopes within that compartment (Karylowski et al., 2004; Lampson et al., 2001). The amount of HA.11 bound HA-GLUT4-GFP in compartments that contain TR or IRAP TR was determined by immunofluorescence of saponin-permeabilized cells.

F) The average results for the distribution of intracellular GLUT4 between endosomes and the specialized compartment determined in four independent experiments ±SD.

the cell-associated HA.11 slowly rose to a level at 480 min that was similar to the plateau reached in control cells at 40 min. This slow rise is the slow exocytosis rate previously measured for GLUT4 at steady state in insulin-stimulated adipocytes expressing DI-AS160 (Zeigerer et al., 2004). The behavior of HA-GLUT4-GFP in the transition from basal to insulin-stimulated conditions in DI-AS160 adipocytes indicated that about 40% of GLUT4 was rapidly recruited to the cell surface, yet this increase in expression of GLUT4 on the PM was not fully maintained in the presence of DI-AS160.

Although the quantitative differences between control and DI-AS160-expressing cells in the transition from basal to insulin-stimulated steady state were readily apparent, this was not reflected in differences in the morphology of the compartments containing HA.11 in the different conditions (Figure S3). Thus, the two pools of GLUT4 detected by the kinetic analysis of DI-AS160-expressing adipocytes were not distinguishable at the level of light microscopy.

Discussion

Here, we establish that the AS160 RabGAP is essential for basal GLUT4 intracellular retention. This is the first demonstration of a protein specifically required for the GLUT4 retention. Knockdown of AS160 results in an acceleration of basal GLUT4 exocytosis, directly demonstrating a role for AS160 as a negative regulator of GLUT4 exocytosis. The effects of AS160 knockdown on GLUT4 are not due to perturbations of general membrane recycling. AS160 knockdown did not change the responsiveness of adipocytes to insulin, since nei-

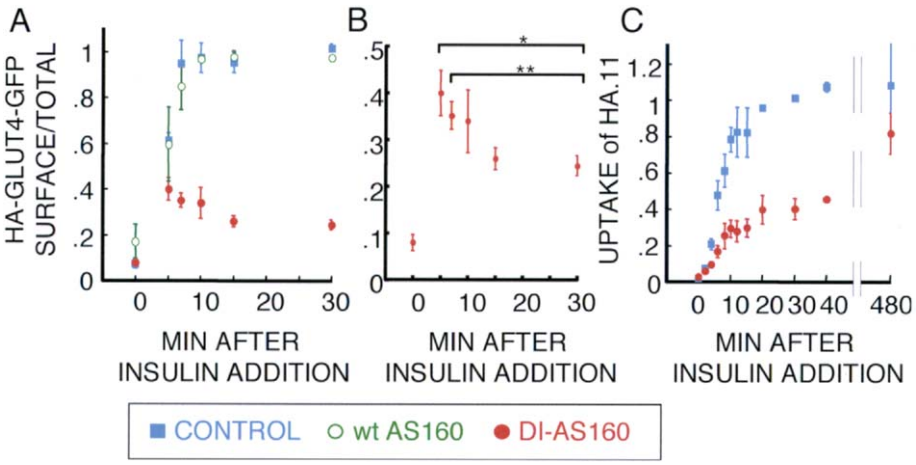

Figure 4. Effect of DI-AS160 on GLUT4 surface expression during the transition from basal to insulin-stimulated state

A) The time course for insulin-stimulated recruitment of HA-GLUT4-GFP into the plasma membrane measured in adipocytes expressing HA-GLUT4-GFP (control) and coexpressing wild-type human AS160, or DI-AS160. The data for the control and DI-AS160 cells are the averages of six experiments ±SEM, and the data for the wild-type AS160 is the average ±SD of two experiments. The values for each experiment were normalized to the plateau level in the control cells of the individual experiments.

B) Data from DI-AS160 in (**A**) on an expanded y axis. The 5 and 7 min points are significantly different from the 30 min point with probabilities of *$p < 0.05$ and **$p < 0.001$, two-tailed, paired t test.

C) Cells were incubated in the presence of insulin and a saturating concentration of HA.11 antibody. At the specified times, the cells were washed, fixed, and total cell-associated HA.11 antibody detected by quantitative immunofluorescence microscopy of permeabilized cells. The data are the averages ±SEM of six experiments, except the 480 min time point, which is the average data from 22 cells measured in a single experiment. The values for each experiment were normalized to surface-to-total ratio of the 30 min time point in the control cells of the individual experiments.

ther the time required to reach a new steady state GLUT4 distribution in insulin nor the concentration of insulin to stimulate translocation were altered. AS160 knockdown had a small but reproducible inhibitory effect on the insulin-stimulated redistribution of GLUT4 into the PM, documenting an unanticipated role for AS160 in maintaining GLUT4 on the surface in insulin-stimulated cells.

The results of studies of both AS160 KD adipocytes and adipocytes expressing the DI-AS160 are consistent with AS160 functioning as a negative regulator of basal GLUT4 exocytosis. In the knockdown cells, a loss of AS160 results in a change in basal retention, as would be expected by loss of a negative regulator of basal exocytosis. On the other hand, expression of the DI-AS160 (whose GAP activity is not inhibited by insulin) has a pronounced affect on the behavior of GLUT4 in insulin-stimulated adipocytes, as anticipated for an unregulated negative regulator of GLUT4 exocytosis (Miinea et al., 2005; Sano et al., 2003; Zeigerer et al., 2004).

Reduction of AS160 also induced a shift in the distribution of GLUT4 from the GLUT4/IRAP compartment toward endosomes. This effect is similar to the effect of insulin on intracellular distribution of GLUT4 in control adipocytes (Bryant et al., 2002; Karylowski et al., 2004). In basal conditions, GLUT4 rapidly cycles between endosomes and the GLUT4/IRAP compartment (Bryant et al., 2002; Karylowski et al., 2004). A plausible explanation for the effect of AS160 KD, as well of that of insulin in wild-type adipocyte cells, is that in the absence of AS160 or the presence of insulin, the rate constant for movement of specialized GLUT4-containing vesicles to and/or their fusion with the PM is markedly increased. As a consequence, the amount of GLUT4 in the specialized compartment decreases, relative to that in the endosome. Based on this explanation, in the basal state AS160 may function to prevent GLUT4-containing vesicle movement and/or fusion with the PM.

Our data establish a role for AS160 in the regulation of GLUT4 insertion into the membrane, although future studies are required to identify the exact site(s) of AS160 action. Identifying the downstream target(s) of AS160 will be important for defining AS160 mode and site(s) of action. In this regard, our demonstration that the phenotype of the knockdown cells was reverted by transient expression of AS160, but not transient expression of an AS160 GAP domain mutant, provides the first direct evidence that AS160 GAP activity is required for GLUT4 retention in adipocytes and thereby provide critical support for the model that the target Rab protein(s) of AS160 regulates GLUT4 movement to the PM. Additional support for the AS160 Rab GAP model comes from the recent report that several Rab proteins are targets of AS160 GAP activity in vitro, although the role of these Rab proteins in GLUT4 trafficking has not been established (Miinea et al., 2005).

Munc18c, a member of the sec1p/Munc18 family of proteins that regulate the assembly of the SNARE fusion complex, has been suggested as a possible target of AS160 regulation (James, 2005). Adipocytes in which Munc18c has been knocked out have a greater sensitivity to insulin; however, unlike the case of AS160 KD, there was no increase in PM GLUT4 in basal Munc18c knockout adipocytes, establishing that Munc18c is not required for basal retention (Kanda et al., 2005). Therefore, it is unlikely that Munc18c is a target of AS160 regulation, since if this were the case Munc18c knockout would have resulted in a similar loss of basal GLUT4 retention, as did AS160 KD.

Is AS160 an obligate intermediate for all of insulin signaling to GLUT4 exocytosis?

The rate of basal GLUT4 exocytosis in AS160 KD adipocytes was three times greater than that in control cells. Still, insulin stimulated a near 4-fold acceleration of GLUT4 exocytosis in the AS160 KD cells, demonstrating an effect of insulin on GLUT4 movement in the knockdown adipocytes. These data clearly establish an important role for AS160 as a negative regulator of GLUT4 trafficking. However, AS160 knockdown did not completely mimic the stimulatory effect of insulin on GLUT4 exocytosis, as would be the case if AS160 was an obli-

gate intermediate for all of insulin signaling to GLUT4 exocytosis. One interpretation of these data is that the partial effect on GLUT4 exocytosis is due to the incomplete knockdown of AS160, with the AS160 remaining in the knockdown adipocytes sufficient to partially, but not completely, inhibit basal GLUT4 exocytosis. An alternative interpretation of the data, which we favor, is that insulin signals to GLUT4 exocytosis through both AS160-dependent and -independent mechanisms. Our observation that the effect of insulin on GLUT4 in AS160 KD cells is inhibited by 100 nM wortmannin indicates that the AS160-independent regulation is also downstream of PI 3 kinase activation.

Several observations argue against the view that the partial loss of retention results from an incomplete knockdown of AS160. If the partial effects on GLUT4 trafficking are due to residual AS160, then the amount of AS160 in the knockdown cells is necessarily limiting. Consequently, the degree of GLUT4 retention would be sensitive to small variations in the amount of AS160 expressed in the individual knockdown cells. This is not what we observed. Within the limits of the sensitivity of the immunofluorescence assay used to quantify AS160 expression per cell, we did not find a linear correlation between the amount of AS160 expressed and the degree of GLUT4 retention in individual cells. This lack of a graded correlation between AS160 and GLUT4 retention is more in line with the proposal of additional AS160-independent regulation of exocytosis.

The studies of DI-AS160, a completely different perturbation of AS160 function, also suggest insulin signaling to GLUT4 exocytosis that is independent of Akt/PKB phosphorylation of AS160. In cells expressing DI-AS160, insulin-stimulated recruitment of GLUT4 to the PM is biphasic, with a rapid recruitment of about 40% of GLUT4 and a slow recruitment of the remaining GLUT4. Finally, in addition to our analysis of AS160, the results of several recent studies also support regulation of GLUT4 exocytosis at multiple steps. Insulin can stimulate a relocalization of GLUT4-containing vesicles to the cell periphery independent of fusion of these vesicles with the PM (Kanda et al., 2005; Semiz et al., 2003; van Dam et al., 2005).

Many studies have documented the complexity of the GLUT4 trafficking pathway. The model emerging from recent studies of GLUT4 trafficking is that its exocytosis is regulated at multiple steps. In a multistep retention mechanism, regulation of sequential steps gives rise to a net GLUT4 exocytosis rate that is slower than any one of the individual steps. Since each of the regulated steps contributes to the net exocytosis rate, the loss of regulation of any one step would not fully recapitulate the effects of insulin on GLUT4 exocytosis. The view that insulin modulates GLUT4 in the PM by altering numerous aspects of this process is mirrored by the molecular complexity of insulin receptor signal transduction. It is possible that different molecular branches of insulin signal transduction intersect GLUT4 trafficking at distinct sites; in which case, signaling to GLUT4 vesicles is not a linear sequence culminating at one site but rather the sum effect of regulation of multiple sites responsible for the basal GLUT4 retention.

Experimental procedures

Ligands and chemicals

Fluorescent antibodies were purchased from Jackson Immunolabs, Inc. (West Grove, Pennsylvania). Anti-GLUT4 antibody was a gift from Sam Cushman. Mouse anti-HA monoclonal antibody (HA.11) was purified from ascites (Covance, Berkley, California) using a protein G affinity column (Amersham, Uppsala, Sweden). The concentration of HA.11 required to saturate the HA-epitope of HA-GLUT4-GFP was determined for each preparation of antibody as previously described (Karylowski et al., 2004). The anti AS160 antibody is an affinity-purified rabbit antibody prepared against a GST fusion protein with a portion of mouse AS160 corresponding to amino acids 584-833 of human AS160.

Cell culture and electroporation

The methods for electroporation have been described (Zeigerer et al., 2002). The HA-GLUT4-GFP and various AS160 constructs have been described (Kane et al., 2002; Lampson et al., 2001; Sano et al., 2003; Zeigerer et al., 2004).

Preparation of shRNA retroviral constructs

The pSiren RetroQ system from BD Clontech Biosciences was used according to the manufacturer's instructions. Amphopak packaging cells were transfected with the retroviral cDNA (BD Clontech Biosciences), and culture medium from the packaging cells, harvested between 24 and 48 hr post-transfection, was used to infect 3T3-L1 cells (Wertheim et al., 2004). The infected cells were selected for growth in puromycin and the surviving cells were pooled, aliquots frozen and some of the cells differentiated, and the behavior of GLUT4 characterized. Pooled knockdown cells were not carried for more than two passages. In the course of this study, multiple batches of both AS160 KD1 and KD2 cells were prepared and we did not find any significant variation in the behavior of GLUT4 among cells derived from different infections.

Kinetic studies

All live cell incubations were performed in serum-free DMEM with 20 mM sodium bicarbonate, 20 mM HEPES (pH7.2) (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 and for measuring the efflux kinetics of HA-GLUT4-GFP have been described in detail elsewhere (Karylowski et al., 2004; Lampson et al., 2001; Zeigerer et al., 2002; 2004).

Fluorescence quantification

All images were collected on a DMIRB inverted microscope using a 40× 1.25 numerical aperture oil immersion objective (Leica Microsystems, Deerfield, Illinois) and a cooled charge-coupled device 12-bit camera (Princeton Instruments, West Chester, Pennsylvania). Image quantification was done as described previously (Dunn et al., 1994; Lampson et al., 2000).

Hexose transport

Hexose transport activity of cells was assayed by the uptake of ^{3}H-2-deoxy-glucose at 37°C, as described (Gibbs et al., 1988).

Distribution between endosomes and the specialized compartment

The distribution of HA-GLUT4-GFP between endosomes and the specialized compartment was determined using a previously described HRP ablation assay (Johnson et al., 1996; Karylowski et al., 2004; Mayor et al., 1998; Zeigerer et al., 2002).

Supplemental data

Supplemental Data include three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/4/263/DC1/.

Acknowledgments

The authors thank members of the McGraw lab, Fred Maxfield, and Tim Ryan for discussions and comments on the manuscript. This work was supported in part by NIH grant DK52852 (T.E.M.) and DK25336 (G.E.L.).

Received: June 3, 2005
Revised: August 25, 2005
Accepted: September 22, 2005
Published: October 11, 2005

References

Bae, S.S., Cho, H., Mu, J., and Birnbaum, M.J. (2003). Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. J. Biol. Chem. *278*, 49530–49536.

Bryant, N., Govers, R., and James, D. (2002). Regulated transport of the glucose transporter GLUT4. Nat. Rev. Mol. Cell Biol. *3*, 267–277.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B., 3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., and Birnbaum, M.J. (2001). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728–1731.

Deneka, M., Neeft, M., and van der Sluijs, P. (2003). Regulation of membrane transport by rab GTPases. Crit. Rev. Biochem. Mol. Biol. *38*, 121–142.

Dunn, K., Mayor, S., Myers, J., and Maxfield, F. (1994). Applications of ratio fluorescence microscopy in the study of cell physiology. FASEB J. *8*, 573–582.

Gibbs, E.M., Lienhard, G.E., and Gould, G.W. (1988). Insulin-induced translocation of glucose transporters to the plasma membrane precedes full stimulation of hexose transport. Biochemistry *27*, 6681–6685.

Govers, R., Coster, A.C., and James, D.E. (2004). Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. Mol. Cell. Biol. *24*, 6456–6466.

Holman, G.D., and Cushman, S.W. (1994). Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. Bioessays *16*, 753–759.

James, D.E. (2005). MUNC-ing around with insulin action. J. Clin. Invest. *115*, 219–221.

Jhun, B.H., Rampal, A.L., Liu, H., Lachaal, M., and Jung, C.Y. (1992). Effects of insulin on steady state kinetics of GLUT4 subcellular distribution in rat adipocytes. Evidence of constitutive GLUT4 recycling. J. Biol. Chem. *267*, 17710–17715.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., and Czech, M.P. (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc. Natl. Acad. Sci. USA *100*, 7569–7574.

Johnson, A.O., Ghosh, R.N., Dunn, K.W., Garippa, R., Park, J., Mayor, S., Maxfield, F.R., and McGraw, T.E. (1996). Transferrin receptor containing the SDYQRL motif of TGN38 causes a reorganization of the recycling compartment but is not targeted to the TGN. J. Cell Biol. *135*, 1749–1762.

Johnson, A.O., Subtil, A., Petrush, R., Kobylarz, K., Keller, S.R., and McGraw, T.E. (1998). Identification of an insulin-responsive, slow endocytic recycling mechanism in Chinese hamster ovary cells. J. Biol. Chem. *273*, 17968–17977.

Kanda, H., Tamori, Y., Shinoda, H., Yoshikawa, M., Sakaue, M., Udagawa, J., Otani, H., Tashiro, F., Miyazaki, J., and Kasuga, M. (2005). Adipocytes from Munc18c-null mice show increased sensitivity to insulin-stimulated GLUT4 externalization. J. Clin. Invest. *115*, 291–301.

Kane, S., Sano, H., Liu, S.C., Asara, J.M., Lane, W.S., Garner, C.C., and Lienhard, G.E. (2002). A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J. Biol. Chem. *277*, 22115–22118.

Kanzaki, M., Mora, S., Hwang, J.B., Saltiel, A.R., and Pessin, J.E. (2004). Atypical protein kinase C (PKC{zeta}/{lambda}) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. J. Cell Biol. *164*, 279–290.

Karylowski, O., Zeigerer, A., Cohen, A., and McGraw, T.E. (2004). GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. Mol. Biol. Cell *15*, 870–882.

Keller, S., Davis, A., and Clairmont, K. (2002). Mice deficient in the insulin-regulated membrane aminopeptidase show substantial decreases in glucose transporter GLUT4 levels but maintain normal glucose homeostasis. J. Biol. Chem. *277*, 17677–17686.

Lampson, M.A., Racz, A., Cushman, S.W., and McGraw, T.E. (2000). Demonstration of insulin-responsive trafficking of GLUT4 and vpTR in fibroblasts. J. Cell Sci. *113*, 4065–4076.

Lampson, M.A., Schmoranzer, J., Zeigerer, A., Simon, S.M., and McGraw, T.E. (2001). Insulin-regulated release from the endosomal recycling compartment is regulated by budding of specialized vesicles. Mol. Biol. Cell *12*, 3489–3501.

Li, D., Randhawa, V.K., Patel, N., Hayashi, M., and Klip, A. (2001). Hyperosmolarity reduces GLUT4 endocytosis and increases its exocytosis from a VAMP2-independent pool in l6 muscle cells. J. Biol. Chem. *276*, 22883–22891.

Martin, S., Tellam, J., Livingstone, C., Slot, J.W., Gould, G.W., and James, D.E. (1996). The glucose transporter (GLUT-4) and vesicle-associated membrane protein-2 (VAMP-2) are segregated from recycling endosomes in insulin-sensitive cells. J. Cell Biol. *134*, 625–635.

Mayor, S., Sabharanjak, S., and Maxfield, F.R. (1998). Cholesterol-dependent retention of GPI-anchored proteins in endosomes. EMBO J. *17*, 4626–4638.

Miinea, C.P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peranen, J., Lane, W.S., and Lienhard, G.E. (2005). AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase activating protein domain. Biochem. J. *391*, 87–93.

Mitra, P., Zheng, X., and Czech, M.P. (2004). RNAi-based analysis of CAP, Cbl, and CrkII function in the regulation of GLUT4 by insulin. J. Biol. Chem. *279*, 37431–37435.

Rosen, E.D. (2002). Molecular mechanisms of adipocyte differentiation. Ann. Endocrinol. (Paris) *63*, 79–82.

Ross, S.A., Herbst, J.J., Keller, S.R., and Lienhard, G.E. (1997). Trafficking kinetics of the insulin-regulated membrane aminopeptidase in 3T3-L1 adipocytes. Biochem. Biophys. Res. Commun. *239*, 247–251.

Rudich, A., and Klip, A. (2003). Push/pull mechanisms of GLUT4 traffic in muscle cells. Acta Physiol. Scand. *178*, 297–308.

Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W., and Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J. Biol. Chem. *278*, 14599–14602.

Semiz, S., Park, J.G., Nicoloro, S.M., Furcinitti, P., Zhang, C., Chawla, A., Leszyk, J., and Czech, M.P. (2003). Conventional kinesin KIF5B mediates insulin-stimulated GLUT4 movements on microtubules. EMBO J. *22*, 2387–2399.

Spang, A. (2004). Vesicle transport: A close collaboration of Rabs and effectors. Curr. Biol. *14*, R33–R34.

Subtil, A., Lampson, M.A., Keller, S.R., and McGraw, T.E. (2000). Characterization of the insulin-regulated endocytic recycling mechanism in 3T3-L1 adipocytes using a novel reporter molecule. J. Biol. Chem. *275*, 4787–4795.

Tanner, L.I., and Lienhard, G.E. (1987). Insulin elicits a redistribution of transferrin receptors in 3T3-L1 adipocytes through an increase in the rate constant for receptor externalization. J. Biol. Chem. *262*, 8975–8980.

van Dam, E.M., Govers, R., and James, D.E. (2005). Akt activation is required at a late stage of insulin-induced GLUT4 translocation to the plasma membrane. Mol. Endocrinol. *19*, 1067–1077.

Watson, R.T., Kanzaki, M., and Pessin, J.E. (2004). Regulated membrane

trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr. Rev. *25*, 177–204.

Wertheim, N., Cai, Z., and McGraw, T.E. (2004). The transcription factor CCAAT/enhancer-binding protein alpha is required for the intracellular retention of GLUT4. J. Biol. Chem. *279*, 41468–41476.

Yang, J., and Holman, G.D. (1993). Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. J. Biol. Chem. *268*, 4600–4603.

Yeh, J.I., Verhey, K.J., and Birnbaum, M.J. (1995). Kinetic analysis of glucose transporter trafficking in fibroblasts and adipocytes. Biochemistry *34*, 15523–15531.

Zeigerer, A., Lampson, M., Karylowski, O., Sabatini, D., Adesnik, M., Ren, M., and McGraw, T. (2002). GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. Mol. Biol. Cell *13*, 2421–2435.

Zeigerer, A., McBrayer, M.K., and McGraw, T.E. (2004). Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. Mol. Biol. Cell *15*, 4406–4415.

Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. *2*, 107–117.