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Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation

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Ishikura S, Klip A. Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am J Physiol Cell Physiol 295: C1016–C1025, 2008. First published August 13, 2008; doi:10.1152/ajpcell.00277.2008.—Insulin causes translocation of glucose transporter 4 (GLUT4) to the membrane of muscle and fat cells, a process requiring Akt activation. Two Rab-GTPase-activating proteins (Rab-GAP), AS160 and TBC1D1, were identified as Akt substrates. AS160 phosphorylation is required for insulin-stimulated GLUT4 translocation, but the participation of TBC1D1 on muscle cell GLUT4 is unknown. Moreover, there is controversy as to the AS160/TBC1D1 target Rabs in fat and muscle cells, and Rab effectors are unknown. Here we examined the effect of knockdown of AS160, TBC1D1, and Rabs 8A, 8B, 10, and 14 (in vitro substrates of AS160 and TBC1D1 Rab-GAP activities) on insulin-induced GLUT4 translocation in L6 muscle cells. Silencing AS160 or TBC1D1 increased surface GLUT4 in unstimulated cells but did not prevent insulin-induced GLUT4 translocation. Knockdown of Rab8A and Rab14, but not of Rab8B or Rab10, inhibited insulin-induced GLUT4 translocation. Furthermore, silencing Rab8A or Rab14 but not Rab8B or Rab10 restored the basal-state intracellular retention of GLUT4 impaired by AS160 or TBC1D1 knockdown. Lastly, overexpression of a fragment of myosin Vb, a recently identified Rab8A-interacting protein, inhibited insulin-induced GLUT4 translocation and altered the subcellular distribution of GTP-loaded Rab8A. These results support a model whereby AS160, Rab8A, and myosin Vb are required for insulin-induced GLUT4 translocation in muscle cells, potentially as part of a linear signaling cascade.

INSULIN STIMULATES THE UPTAKE of glucose from the circulation into muscle and fat tissues. At the cellular level, this is brought about through a gain in glucose transporter 4 (GLUT4) at the cell surface. The L6 myogenic and 3T3-L1 adipogenic cell lines are well-characterized cell systems used to understand this process. Fundamental aspects of GLUT4 traffic are conserved between the two systems, e.g., partial segregation of GLUT4 from constitutively recycling endosomes, stimulation of GLUT4 exocytosis by insulin through signaling by phosphatidylinositol 3-kinase and Akt, and VAMP2-mediated fusion (33, 39). However, differences are also noted, e.g., insulin decreases GLUT4 endocytosis only in fat cells, and the hormone induces a larger gain in surface GLUT4 in 3T3-L1 cells compared with L6 cells. In this regard, these lines resemble the behavior of primary adipocytes and skeletal muscles, respectively.

Intense effort has been devoted to identifying Akt targets and subsequent events that should complete the signaling cascade that regulates GLUT4 vesicle traffic. In this regard, the Rab-GTPase-activating protein (Rab-GAP) TBC1D4 was renamed AS160 when it was found to be an Akt substrate of 160 kDa (13). AS160 is phosphorylated in muscle and fat cells in response to insulin (2, 13). Studies in both adipocytes and muscle cells transfected with AS160 mutants conclude that AS160 phosphorylation is required for its signaling toward downstream effectors and that AS160 phosphorylation is coupled to inactivation of its Rab-GAP activity (27, 32, 41). Hence AS160 can be considered to be a brake that is removed by upstream signaling. Accordingly, AS160 knockdown in adipocytes causes an insulin-independent rise in surface GLUT4 (6). No such information is available for muscle cells.

In addition, skeletal muscle expresses significant high levels of TBC1D1, a protein with homology to AS160 that is more scarce in adipose tissue (3, 31). In 3T3-L1 adipocytes, TBC1D1 knockdown did not affect surface GLUT4 levels in either the unstimulated or insulin-stimulated states (3), suggesting that endogenous levels of TBC1D1 are not as relevant as AS160 in those cells, even though overexpression of wild-type TBC1D1 inhibited insulin-induced GLUT4 translocation and TBC1D1 is also phosphorylated upon insulin stimulation (3, 31). The higher expression of TBC1D1 in muscle begs of analysis of its contribution to GLUT4 traffic in this tissue.

The TBC domains of AS160 and TBC1D1 have in vitro Rab-GAP activity toward the same cohort of Rab small G proteins, namely, Rabs 2A, 8A, 8B, 10, and 14 (18, 23). We recently reported that overexpression in L6 muscle cells of wild-type Rab8A or Rab14, but not Rab10, can rescue the inhibition of GLUT4 translocation caused by an AS160 mutant in which four of the Akt phosphorylation sites are mutated to Ala (AS160–4P) with putative constitutively active Rab-GAP activity (11). On the other hand, through a different strategy, i.e., gene expression silencing, Rab10 was implicated in insulin-dependent GLUT4 translocation in 3T3-L1 adipocytes, and not Rabs 8A, 8B, or 14 (26, 28). These divergent conclusions for muscle and adipose cells may be due to cell-specific differences or to the different strategies employed, a matter that calls for further scrutiny. Moreover, in neither instance is the mechanism whereby the individual Rabs impinge on GLUT4 vesicle traffic known.

The objective of the present study was to explore the events downstream of AS160 and TBC1D1 that may regulate GLUT4 traffic in muscle cells. First, by silencing expression of AS160, TBC1D1, Rabs 8A, 8B, 10, and 14, we show that not only AS160 but also TBC1D1 is required for intracellular retention of GLUT4 in myoblasts or myotubes, two insulin-responsive...
stages in the differentiation of these cells. Second, we provide further support for the selective participation of Rab8A and Rab14 in GLUT4 translocation. Third, given the recent discovery that Rab8A interacts with myosin Vb (25), we explore the contribution of this motor protein to GLUT4 traffic. The results support a model whereby AS160, Rab8A, and myosin Vb are required for insulin-regulated GLUT4 translocation in muscle cells, potentially as part of a linear signaling cascade.

**MATERIALS AND METHODS**

Reagents, small inhibitory RNAs, and constructs. o-Phenylenediamine, monoclonal anti-actin 1 antibody (mouse IgM isotype, clone BM-75.2), and monoclonal anti-FLAG antibody were from Sigma-Aldrich (Oakville, ON, Canada). Polyclonal anti-myc antibody (A-14) was from Santa Cruz (Santa Cruz, CA). Polyclonal anti-AS160 antibody was from Upstate (Lake Placid, NY). Monoclonal anti-Rab8 and polyclonal anti-Rab14 antibodies were from BD Biosciences (Mississauga, ON, Canada) and Abcam (Cambridge, MA), respectively. Indocarbocyanine (Cy3)-bound secondary antibody was from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-bound goat anti-mouse and anti-rabbit IgG antibodies or donkey anti-mouse IgM antibody were from Jackson ImmunoResearch Laboratories (West Grove, PA). Monoclonal anti-glut4 antibody (1F8) was from R&D Systems (Minneapolis, MN). Monoclonal anti-Akt, anti-phospho-Akt (Thr308), and anti-phospho-Akt (Ser473) antibodies were from Cell Signaling Technology (Danvers, MA). Polyclonal anti-Rab10 antibody was generously provided by Dr. G. E. Lienhard (Dartmouth Medical School, Hanover, NH) (26).

Small inhibitory RNAs (siRNAs) targeted against AS160 (siAS160-TGG AGC CAA GGT GAT ATT CTA-), TBC1D1 (siTBC1D1-TCC TTC GAT AAC GAC TCT GAA-), Rab8A (siRab8A-CAG GCC GAA GGC CAA CAT CAA-), Rab8B (siRab8B-AAC GAT AGA ACA CGG AAA-A), Rab10 (siRab10-GGG CAT CAT GCT AGT GTA TGA-), Rab14 (siRab14-AAG GAA CCT CAC CAA CCC AAA-A), and nonrelated control (siNRAT-AAT AAC AAG GCT ATG AAG AGA TAC-) were purchased from Qiagen (University of British Columbia, Vancouver, BC, Canada) (17). To make FLAG-tagged MVb-Tail expression vector, cDNA corresponding to the entire region of coding sequence was sequenced by DNA sequencing.

Green fluorescent protein (GFP)-tagged wild-type Rab8A (GFP-Rab8A-WT), constitutive active Rab8A with mutation Thr22 to Asn (GFP-Rab8A-DN, GDP-locked) were the same as used by Hamra et al. (8). The threshold cycle (Ct) was generated by the \\DeltaΔCt method against 18S rRNA.

**Cell lysates and immunoblotting.** Cells were lysed as described previously (20), and equal amounts of protein samples were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and immunoblotted with the following antibodies: anti-AS160 (1:1,000), anti-actin 1 (1:5,000), anti-Rab8 (1:1,000), anti-Rab10 (1:50), anti-Rab14 (1:2,000), anti-glut4 (1:1,000), anti-actinin (1:5,000), anti-phospho-Akt (Thr308) (1:5,000), and anti-phospho-Akt (Ser473) (1:5,000). Primary antibodies were detected with the appropriate HRP-conjugated species-specific IgG antibodies. Detection was completed with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) and HRP Blot CL autoradiography film from Denville Scientific (Metuchen, NJ).

**Cell-surface GLUT4myc detection by an absorbance-based assay.** Cell-surface GLUT4myc in siRNA-transfected cells was detected by colorimetric assay as described previously (36, 37). Briefly, cells grown in 24-well plates and serum starved 3 h were treated with or without 100 nM insulin for 20 min. Then cells were washed twice with ice-cold PBS, blocked 10 min with 5% (vol/vol) goat serum, and reacted with polyclonal anti-myosin myc antibody (1:200) for 1 h at 4°C. After being washed 10 times with PBS, cells were fixed 10 min with 3% (vol/vol) paraformaldehyde in PBS, quenched 10 min with 50 mM glycine in PBS, and reacted with HRP-bound goat anti-rabbit secondary antibody (1:2,000) for 1 h at 4°C. The cells were washed 10 times with PBS, incubated with 1.0 ml 0.1 M Thiamine, and allowed to develop for 20–30 min in the linear range, at room temperature. The reaction was stopped with 0.25 ml per well of 3 M HCl. Supernatants were collected and absorbance was measured at 492 nm. Background absorbance obtained in the absence of anti-myosin myc antibody was subtracted from all values.

**Fluorescent detection of Rab8A mutants, Rab11A, and myosin Vb fragments.** Fluorescence microscopy was used to detect GFP-Rab chimera and FLAG-tagged myosin fragments that were cotransfected in the same cells. Antigen-specific immunostaining was performed as described previously (14) but using an anti-FLAG primary antibody. In brief, cells were fixed, quenched, permeabilized in 0.1% (vol/vol) Triton X-100 for 20 min, blocked with 5% skim milk, and labeled with monoclonal anti-FLAG (1:5,000) in 5% skim milk. After being washed, cells were incubated with Cy3-coupled secondary antibody (1:1,000) for 1 h at room temperature. GFP fluorescence was detected in parallel. To reduce possible fluorophore spectral crossover, cells were imaged by multichannel scanning with a Zeiss LSM 510 laser-scanning confocal microscope. Images of the 200-μm optical slice in the central plane of the cells are shown, so as to focus on detection in the perinuclear region.

**Cell-surface GLUT4myc detection by immunofluorescence microscopy.** L6-Glut4myc cells were transfected with GFP chimera of myosin Vb-tail or FLAG-tagged myosin Vb-GT. After 24 h, cells were serum starved 3 h, and surface GLUT4myc following treatment with or without insulin (100 nM, 20 min) was detected by indirect immunofluorescence microscopy in nonpermeabilized cells as described previously (32). Fluorescent images were obtained with a Zeiss LSM 510 laser-scanning confocal microscope. Whole cells were scanned along the z-axis, and a single composite image (collapsed xy projection) of the optical cuts per cell was generated using LSM510.
Image software. Pixel intensity of single cells (20–30 cell per condition, each repeated in 3 independent experiments) was quantified by ImageJ software (National Institutes of Health, Bethesda, MD). Raw data were converted to fold-change above basal levels relative to surface GLUT4myc signal in untransfected, vicinal cells (controls).

Statistical analysis. Statistical analyses were carried out using Prism 4.0 software (San Diego, CA). Two groups were compared using Student’s t-test, and more than two groups were compared using analysis of variance with Tukey’s post hoc analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Silencing AS160 increases basal surface GLUT4myc but does not prevent insulin-induced GLUT4myc translocation. Previously, we demonstrated that transient expression of the mutant AS160-4P markedly inhibited insulin-stimulated GLUT4 translocation in L6-GLUT4myc myoblasts (32). To further assess the role of AS160 in insulin-regulated GLUT4 traffic, we knocked down AS160 expression in this cultured muscle cell system using siRNA. Endogenous AS160 protein expression was reduced by ~70% with AS160 siRNA (siAS160) in myoblasts and myotubes, compared with cells transfected with nonrelated siRNA (siNR) (Fig. 1A).

In cells transfected with siNR, insulin raised surface GLUT4myc levels by approximately 2.6-fold in myoblasts and myotubes, compared with unstimulated (basal) cells (Fig. 1B). AS160 knockdown per se caused an elevation in basal levels of surface GLUT4myc compared with basal siNR-transfected cells (Fig. 1B). This effect was larger in myoblasts (1.6-fold) than in myotubes (1.3-fold). In insulin-stimulated myoblasts, AS160 knockdown allowed an additional rise in surface GLUT4myc, attaining a slightly higher level (20%) than the response in insulin-stimulated siNR-transfected cells. A similar tendency to elevate the insulin response was observed in insulin-stimulated myotubes treated with siAS160, but the difference did not attain statistical significance (Fig. 1B).

Therefore, the net gains in cell surface GLUT4myc above basal values were basically unaffected by AS160 knockdown in myoblasts or myotubes (Fig. 1B, inset). These data suggest that AS160 is required primarily for intracellular retention of GLUT4 in L6 muscle cells, such that reducing its expression increased the basal levels of surface GLUT4myc but did not prevent the insulin response. Moreover, AS160 appears to be a more effective intracellular retainer of GLUT4 in myoblasts than in myotubes.

Expression of Rabs 8A, 8B, 10, and 14, which are in vitro substrates of AS160 Rab-GAP activity, and the expression or phosphorylation levels of Akt in the basal or insulin states were unaltered in L6 cells treated with AS160 siRNA (data not shown). In addition, AS160 knockdown did not affect total GLUT4 expression levels (Supplemental Fig. 1; supplemental material for this article is available online at the American Journal of Physiology-Cell Physiology website). Hence, the increase of surface GLUT4 in basal cells was not due to altered expression of its targets or GLUT4 nor to inappropriate activation of Akt.

TBC1D1 knockdown increases basal surface GLUT4myc and potentiates insulin-induced GLUT4 translocation in myotubes. The expression of TBC1D1 was determined by real-time quantitative PCR and was calculated relative to 18S rRNA since a TBC1D1 antibody was not available. TBC1D1 mRNA was reduced by ~62% with TBC1D1 siRNA (siTBC1D1) in myoblasts or myotubes, compared with cells transfected with siNR (Fig. 2A). Conversely, siAS160 did not affect TBC1D1 mRNA levels. In addition, the simultaneous knockdown of AS160 and TBC1D1 did not reduce TBC1D1 mRNA levels beyond the effect attained by siTBC1D1 alone.

In myoblasts, silencing TBC1D1 caused a slight increase in basal and insulin-stimulated surface GLUT4myc levels that did not, however, attain statistical significance (Fig. 2B). Accord-
over, TBC1D1 knockdown in myotubes compounded the insulin-dependent gain in surface GLUT4 myc by an additional 50% compared with insulin-stimulated siNR-transfected counterparts. Hence, in myotubes, TBC1D1 knockdown potentiated insulin action, such that the net gain above basal in surface GLUT4 was increased from 1.7 units in siNR-treated cells to 2.6 units in siTBC1D1-treated cells (Fig. 2B, inset).

In either myoblasts or myotubes, double-knockdown of TBC1D1 and AS160 caused no further elevation of basal surface GLUT4 content than that caused by siAS160 (Figs. 1B and 2B). Thus, like AS160, TBC1D1 is required for intracellular retention of GLUT4 in muscle cells; but in direct contrast with AS160, this function of TBC1D1 is more prominent in myotubes than in myoblasts. Further, TBC1D1 knockdown compounded insulin-induced GLUT4 translocation in myotubes, suggesting that TBC1D1 may normally participate to restrain the increase of surface GLUT4 elicited by insulin in myotubes.

Silencing Rab8A or Rab14 inhibits insulin-induced GLUT4 translocation. As stated in the Introduction, overexpression of wild-type Rab8A or Rab14 prevented the drop in insulin-stimulated GLUT4 translocation caused by AS160-4P, whereas Rab10 was unable to rescue this inhibitory action in L6 myoblasts (11). These results suggested that Rab8A and Rab14 are downstream effectors of AS160 in muscle cells. In contrast, Rab10 knockdown but not silencing of either Rabs 8A, 8B, or 14 reduced GLUT4 translocation in 3T3-L1 adipocytes (26, 28). Those results pointed to Rab10 as an important molecule in insulin-dependent GLUT4 traffic. It may be concluded that either cell type-specific Rabs participate in GLUT4 traffic or the different strategies used in the two cell lines are responsible for the divergent results. To discern between these possibilities, we knocked down the expression of each Rab protein individually or in combination in L6 muscle cells.

Assessing the efficacy of Rabs 8A and 8B siRNAs to silence the expression of their respective proteins was complicated because isoform-specific antibodies are not available. Rab8A siRNA reduced by 45% the total amount of protein recognized by anti-Rab8 antibody, and the decrease was mostly in the upper portion of the immunoreactive band (Fig. 3A). In contrast, Rab8B siRNA markedly eliminated the lower portion of the Rab8 immunoreactive band and reduced the total amount of protein recognized by anti-Rab8 antibody by 50%. Cotransfection of Rabs 8A and 8B siRNAs reduced the total Rab8 immunoreactivity by 80% (Fig. 3A). Hence, the protein band recognized by anti-Rab8 antibody in siNR-transfected cells is most likely a doublet of Rabs 8A and 8B, and siRNAs to Rabs 8A and 8B can specifically knock down their respective proteins. The siRNAs to Rabs 10 and 14 reduced their respective endogenous proteins by 70% and 85%, respectively (Fig. 3A).

We next determined the effects of individual Rab knockdown on surface GLUT4 myc levels in myoblasts and myotubes. There was no effect on basal-state GLUT4 myc levels on knockdown of any of the Rabs tested (Fig. 3B). On the other hand, Rab8A knockdown reduced the net insulin-dependent gain in surface GLUT4 myc by 65% compared with siNR-
transfected myoblasts (Fig. 3B). Rab14 knockdown also reduced the net gain in surface GLUT4myc by 30% compared with siNR-transfected myoblasts. Cotransfection of Rabs 8A and 14 siRNAs did not produce additive inhibition of insulin-induced GLUT4myc translocation (Fig. 3C). In contrast, neither Rab8B nor Rab10 knockdown exerts any significant effect on GLUT4myc translocation (Fig. 3B). Similar results were also observed in myotubes (data not shown). Silencing of any of these Rabs had no consequence on upstream insulin signaling, since phosphorylation or expression of Akt was unaffected by knockdown of any of the Rabs tested in the present study (Fig. 3D). As well, knockdown of these Rabs did not affect the expression levels of GLUT4 (Supplemental Fig. 1) or insulin-induced actin remodeling (data not shown). Collectively, these data indicate that Rabs 8A and 14, but not Rabs 8B and 10, are involved in insulin-regulated GLUT4 translocation in L6 myoblasts and myotubes.

Knockdown of Rab8A or Rab14 restores basal retention of GLUT4myc impaired by AS160 or TBC1D1 knockdown. To further determine which Rab(s) is involved in GLUT4 traffic downstream of AS160 and TBC1D1 in muscle cells, we tested the ability of individual Rab knockdown to restore the basal levels of GLUT4 that had been elevated by AS160 or TBC1D1 gene silencing.

Cotransfection of AS160 siRNA and siRNA to each Rab was performed in myoblasts because the effect of AS160 knockdown on the basal surface GLUT4 was more marked in myoblasts than in myotubes (Fig. 1B). In AS160 knockdown cells, siRNAs to Rabs effectively knocked down their respective target proteins (Fig. 4A). Only Rab8A siRNA reversed the ability of AS160 siRNA to increase basal surface GLUT4myc content (Fig. 4C). Essentially, double-knockdown of Rab8A and AS160 neutralized each others’ effects, so that the basal surface GLUT4myc were comparable to those in siNR-treated cells. In addition, in double-knockdown experiments, Rab14 siRNA showed a tendency to counter the effect of AS160 knockdown on basal surface GLUT4myc, but this did not reach statistical significance (Fig. 4C). Lastly, neither Rab8B nor Rab10 silencing had any significant effect on basal surface GLUT4myc in cells depleted of AS160. These data support the hypothesis that Rab8A and possibly Rab14 are targets of AS160 in L6 myoblasts.

In contrast to AS160, the TBC1D1 downstream Rab(s) leading to GLUT4 traffic are unknown, although the TBC domains of both proteins show similar Rab substrate specificity in vitro. To determine which Rab(s) is involved in GLUT4 traffic downstream of TBC1D1, we adapted the same strategy used for AS160. Cotransfection of TBC1D1 siRNA and siRNA to each Rab was performed in myotubes because the effect of TBC1D1 knockdown on the basal surface GLUT4 was larger in myotubes than in myoblasts (Fig. 2B). In TBC1D1 knockdown cells, siRNAs to Rabs effectively knocked down their respective target proteins (Fig. 4B). As predicted, the increase in basal surface GLUT4 caused by TBC1D1 knockdown was reversed by Rab8A knockdown (Fig. 4D). Interestingly, Rab14
knockdown also restored the effect of TBC1D1 knockdown on basal surface GLUT4 \textit{myc} (Fig. 4D). The level of basal surface GLUT4 in TBC1D1/Rab8A or TBC1D1/Rab14 double-knockdown cells was comparable to that in siNR-treated cells. On the other hand, silencing Rab8B or Rab10 had no effect on basal surface GLUT4 \textit{myc} in TBC1D1-depleted cells (Fig. 4D).

These data suggest that Rab8A and Rab14 are both targets of TBC1D1 in L6 myotubes.

\textbf{Myosin Vb-tail influences GTP-loaded Rab8A localization and inhibits insulin-induced GLUT4\textit{myc} translocation.} In the active, GTP-bound form, Rabs interact with a large number of specific effector proteins to carry out their physiological roles. Recently, myosin Vb, a member of myosin V unconventional motor proteins family, was found to interact with Rab8A and Rab11A at different sites (25). Interestingly, myosin Va, another member of the myosin V family, participates in GLUT4 translocation in 3T3-L1 adipocytes (38).

To explore the interaction of myosin Vb with Rab8A and Rab11A in L6 muscle cells, we coexpressed GFP-Rab8A or GFP-Rab11A along with FLAG-MVb-Tail or FLAG-MVb-GT and examined their subcellular distributions by confocal laser microscopy. On its own, FLAG-MVb-tail distributed as a cluster of perinuclear puncta (Fig. 5A). GFP-Rab8A-WT alone showed a preferential perinuclear distribution and was also present over areas of the cytosol (Fig. 5B). Coexpression of FLAG-MVb-tail markedly altered the distribution of GFP-Rab8A-WT, concentrating it in the perinuclear area and somewhat depleting it in the cytosol, making it to resemble the distribution of FLAG-MVb-tail (Fig. 5B). Not surprisingly, GFP-Rab8A-WT completely colocalized with FLAG-MVb-tail in the perinuclear puncta. In contrast to FLAG-MVb-tail, FLAG-MVb-GT did not change distribution of GFP-Rab8A-WT (Fig. 5B). Transfected GFP-Rab8A-CA alone showed a similar distribution pattern to that of GFP-Rab8A-WT (Fig. 5B), and coexpression of FLAG-MVb-tail changed the distribution of GFP-Rab8A-CA similar to its effect on GFP-Rab8A-WT.
Interestingly, GFP-Rab8A-DN did not show perinuclear localization and instead it distributed all over the cytosol (Fig. 5B). Coexpression of FLAG-MVb-tail did not alter the distribution of GFP-Rab8A, whereas both myosin fragments alter Rab11A. A: schematic illustration of constructs of COOH-terminal fragment of rat myosin Vb (MVb-tail) or the globular tail of mouse myosin Vb (MVb-GT). Mouse MVb-GT has 97% amino acid identity with the corresponding sequences in rat MVb-tail.

B: L6-GLUT4myc myoblasts were transiently transfected with the indicated green fluorescent protein (GFP)-tagged Rab8A wild-type (Rab8A-WT), constitutively active (Rab8A-CA), or dominant-negative (Rab8A-DN) and GFP-Rab11A wild-type (Rab11A-WT) expression vectors alone or with FLAG-MVb-tail or FLAG-MVb-GT, as indicated. Cells were serum starved for 3 h and stained with anti-FLAG antibody as described in MATERIALS AND METHODS. Images were obtained by confocal laser microscopy. Representative images of three independent experiments are shown. Perinuclear images are 3 times magnified relative to the other images. Bars, 10 μm.

Fig. 5. Myosin Vb-tail, but not myosin Vb-globular tail (Vb-GT), alters the distribution of GTP-loaded Rab8A, whereas both myosin fragments alter Rab11A. A: schematic illustration of constructs of COOH-terminal fragment of rat myosin Vb (MVb-tail) or the globular tail of mouse myosin Vb (MVb-GT). Mouse MVb-GT has 97% amino acid identity with the corresponding sequences in rat MVb-tail. B: L6-GLUT4myc myoblasts were transiently transfected with the indicated green fluorescent protein (GFP)-tagged Rab8A wild-type (Rab8A-WT), constitutively active (Rab8A-CA), or dominant-negative (Rab8A-DN) and GFP-Rab11A wild-type (Rab11A-WT) expression vectors alone or with FLAG-MVb-tail or FLAG-MVb-GT, as indicated. Cells were serum starved for 3 h and stained with anti-FLAG antibody as described in MATERIALS AND METHODS. Images were obtained by confocal laser microscopy. Representative images of three independent experiments are shown. Perinuclear images are 3 times magnified relative to the other images. Bars, 10 μm.

Fig. 6. In untransfected, control cells, insulin elevated surface GLUT4myc levels by 2.0-fold. Expression of FLAG-tagged MVb-GT did not affect surface GLUT4myc levels in basal cells, or the insulin response of GLUT4 translocation. On the other hand, basal surface GLUT4myc levels were 20% lower in basal cells expressing a GFP-chimera of MVb-tail compared with untransfected cells. More importantly, GFP-MVb-tail significantly inhibited insulin-induced GLUT4 translocation by 40%. Similar results were obtained in one experiment using FLAG-tagged MVb-tail (30–50 cells analyzed per condition). Expression of this construct reduced the insulin response from 2.1-fold to 1.3-fold, whereas expression of FLAG-MVb-GT in parallel again failed to reduce the response to the hormone. This result confirmed the different action of MVb-tail and MVb-GT when expressed at comparable levels (determined by immunoblotting the FLAG epitope; results not shown). In neither circumstance did the total levels of GLUT4 change as determined by immunoblotting (Supplemental Fig. 1).
untransfected cells within a given treatment. For infected cell/H11006 AS160 increased basal surface levels of GLUT4 toward the cell surface. AS160 or TBC1D1 would allow release of GLUT4 vesicles halt GLUT4 vesicle traffic, until they are deactivated by shift their target Rabs toward the inactive state, and thereby to myosin constructs on GLUT4, given that MVb-GT interacts Rab11A, this interaction cannot explain the action of the Rab8A. In contrast, although myosin Vb can also interact with myosin Vb impinges on GLUT4 traffic via its interaction with domain. Because active Rab8A localization is also selectively altered by MVb-tail but not by MVb-GT, it is likely that myosin Vb impinges on GLUT4 traffic via its interaction with Rab8A. In contrast, although myosin Vb can also interact with Rab11A, this interaction cannot explain the action of the myosin constructs on GLUT4, given that MVb-GT interacts with Rab11A but does not affect the transporer.

DISCUSSION

AS160 and TBC1D1. AS160 and TBC1D1 are predicted to shift their target Rabs toward the inactive state, and thereby to halt GLUT4 vesicle traffic, until they are deactivated by phosphorylation. Accordingly, we hypothesized that silencing AS160 or TBC1D1 would allow release of GLUT4 vesicles toward the cell surface.

Here we show that siRNA-mediated gene silencing of AS160 increased basal surface levels of GLUT4myc in L6 muscle cells (Fig. 1), similar to observations in 3T3-L1 adipocytes (6). In contrast, while silencing AS160 reduced the insulin response of GLUT4 in 3T3-L1 adipocytes (6), this strategy did not prevent insulin-induced GLUT4 translocation in L6 muscle cells (Fig. 1). This behavior in L6 muscle cells is compatible with AS160 functioning as a stop signal that is relieved upon phosphorylation by Akt in response to insulin. Absence of the stop signal would not be expected to prevent insulin signaling to bring about GLUT4 translocation. AS160 may have other functions in the insulin-stimulated state in 3T3-L1 adipocytes.

Silencing TBC1D1 elevated basal surface GLUT4 levels in L6 myotubes, indicating that endogenous TBC1D1 is also required for intracellular retention of GLUT4 in muscle cells. Consistent with this, overexpression of wild-type TBC1D1 in 3T3-L1 adipocytes lowered the basal levels of surface GLUT4 (3). The consequence of TBC1D1 silencing on surface GLUT4 was more notable in myotubes than in myoblasts. Consistent with this result, Chavez et al. (3) showed that TBC1D1 expression is induced during the differentiation of L6 cells. On the other hand, depletion of TBC1D1 even augmented insulin-induced GLUT4 translocation in L6 myotubes (Fig. 3B), suggesting that TBC1D1 restrains insulin-stimulated GLUT4 progression. Consistent with this concept, wild-type TBC1D1 overexpression inhibited insulin-induced GLUT4 translocation in 3T3-L1 adipocytes (3), whereas wild-type AS160 has no effect on insulin-induced GLUT4 translocation in either L6 muscle cells or 3T3-L1 adipocytes (27, 32). Furthermore, TBC1D1 phosphorylation by Akt did not inhibit its Rab-GAP activity (3), suggesting that TBC1D1 participation in GLUT4 traffic is not regulated via this kinase. Instead, TBC1D1 activity appears to be regulated by AMP-activated protein kinase (AMPK) (3), and indeed AMPK and Akt phosphorylate TBC1D1 on different sites (4). These results along with our findings suggest that TBC1D1 is involved in GLUT4 traffic in muscle cells but its Rab-GAP activity is insulin independent and may instead be controlled by AMPK, conceivably during muscle contraction.

Silencing both AS160 and TBC1D1 did not produce additive effects on basal surface GLUT4 levels, and, although elevated, these levels did not match the stimulation attained with insulin. These results suggest that removal of stop signals is essential for insulin-induced GLUT4 translocation, but not sufficient to produce a maximal response to the hormone. Other signals, such as the activation of guanine nucleotide exchange factors for downstream Rabs, and Rac activation causing actin remodeling, may be required for maximal GLUT4 translocation.

Rabs 8A, 8B, 10, and 14. As mentioned in the Introduction, the identity of the Rab targets of AS160 in muscle and fat cells is controversial. Furthermore, the Rab targets of TBC1D1 remain unknown. Hence, we here tested the same gene-silencing strategies used in 3T3-L1 adipocytes to identify whether the discrepancy arises from cell-specific differences or from the different experimental approaches, and which Rabs are targets of TBC1D1. Concerted knockdown of Rab8A along with knockdown of either AS160 or TBC1D1 restored the levels of surface GLUT4 that had been elevated by AS160 or TBC1D1 knockdown (Fig. 4). Concerted knockdown of Rab14 and AS160 had a similar tendency that did not achieve statistical significance, whereas Rab14 knockdown restored the rise in surface GLUT4 evoked by TBC1D1 knockdown. In contrast, Rab8B and Rab10 knockdown did not revert the action of AS160 or TBC1D1 depletion. These observations buttress the concept that Rab8A and probably Rab14 act downstream of AS160 and TBC1D1 in muscle cells. Clearly, these results contrast with the observations made in 3T3-L1 adipocytes and suggest that cell-specific differences account for the preferen-

![Image](https://example.com/image.png)
tial participation of Rab10 downstream of AS160 in those cells.

As shown in Fig. 3, knockdown of any individual Rab or in combination was inconsequential on surface GLUT4myc levels in the basal state. These results are consistent with the retaining action of AS160 or TBC1D1, indicating that inactive Rabs or absence of Rabs precludes escape of GLUT4 vesicles toward the cell surface in unstimulated cells. In contrast, selective knockdown of Rab8A or Rab14, but not of Rab8B or Rab10, markedly diminished the insulin-dependent gain in surface GLUT4myc in both myoblasts and myotubes. These results suggest that Rab8A and Rab14, presumably as active Rabs, are required for insulin-induced GLUT4 translocation in muscle cells.

Although Rab8A depletion affected stress fiber formation in HT1080 fibrosarcoma cells (10), it did not affect actin filaments in either the basal or insulin-stimulated muscle cells (data not shown). This finding suggests that the concomitant reduction in insulin-dependent GLUT4 translocation is not due to interference with actin remodeling, a function that also contributes to the net gain in surface GLUT4 (34). However, as discussed below, it is possible that Rab8A acts through myosin Vb, which in turn interacts with actin filaments. Of note, in diverse cell types, Rab8A localizes to both the trans-Golgi network (TGN) and the cell periphery, and it regulates transport of membrane proteins (1, 7). Rab14 also localizes to the TGN and endosomes and mediates transport between endosomes and between TGN and endosomes (9, 12, 15, 22). Further studies should explore the precise locus of action of Rab8A and Rab14 on GLUT4 traffic in muscle cells. Myosin Vb. The regulatory action of Rab8A in GLUT4 traffic may be revealed from its interacting proteins. By yeast two-hybrid and fluorescence resonance energy transfer analysis in HeLa cells, myosin Vb was found to interact with Rab8A and Rab11A at different sites (25). Interestingly, several Rab family members are proposed regulators of myosin function in organelar transport, and processive myosins such as myosin V are effectors of Rabs in the delivery of vesicular cargo (29). In addition, Rab11A is involved in GLUT4 transit through the recycling endosome (40), although AS160 and TBC1D1 do not show Rab-GAP activity toward Rab11A (18, 23). Hence, we explored whether myosin Vb could be functionally linked to Rab8A (possibly as its effector) or to Rab11A in the pathway of GLUT4 traffic in muscle cells, taking advantage of the identified interacting segment in myosin Vb. Expression of this myosin Vb tail segment (MVb-tail) altered the distribution of both GFP-Rab8A or GFP-Rab11A in the muscle cells (Fig. 5), akin to observations made in HeLa cells (25), and inhibited insulin-dependent GLUT4 translocation to the cell surface (Fig. 6). On the other hand, MVb-GT, which altered distribution of Rab11A but not Rab8A, was inconsequential on surface GLUT4 levels, suggesting that the inhibition of GLUT4 translocation caused by MVb-tail is related to Rab8A but not Rab11A. Hence, it is conceivable that myosin Vb is a Rab8A effector leading to GLUT4 mobilization in muscle cells. Although Rab8A can also interact with myosin Vc (24), we could not detect expression of this isoform in L6 cells at the myoblast stage (result not shown).

Myosins Va and Vb are efficient processive motors, and the myosin Va movement in 37-nm steps corresponds with the helical periodicity displayed by actin filaments (5, 35). These observations suggest that these myosins are ideal candidates to transport vesicular cargo along actin tracks. Importantly, a dynamic actin cytoskeleton is required for GLUT4 traffic (21). Future work should reveal whether myosin V lies at the convergence point between the two insulin-derived signaling arms, which respectively involve Rac-dependent actin dynamics and Akt-dependent AS160 inactivation toward Rab8A (39).

In conclusion, we present evidence that GLUT4 is intracellularly retained by mechanisms involving AS160 preferentially in myoblasts and TBC1D1 in myotubes. Second, that Rab8A is a bona fide target of AS160, and Rab8A and Rab14 are targets of TBC1D1 in myotubes, for the purpose of GLUT4 retention. This points to a cell type-specific difference with adipocytes, which engage Rab10 in this function. Third, the relocalization of Rab8A by a Rab8A-interacting fragment of myosin Vb and concomitant inhibition of GLUT4 translocation suggests that interaction of full-length myosin Vb with Rab8A may be required for this process. Overall, the results support a model whereby AS160, Rab8A, and myosin Vb are required for insulin-induced GLUT4 translocation in muscle cells. On the basis of the emerging paradigm that organelle-linked Rab proteins associate with effector myosin molecules (29), we hypothesize that AS160/TBC1D1 act on Rab8A, which in turn interacts with myosin Vb to achieve GLUT4 translocation to the membrane.

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DISCLOSURES

We do not have any conflict of interest to report.

REFERENCES


