GLUT4 Vesicle Recruitment and Fusion Are Differentially Regulated by Rac, AS160, and Rab8A in Muscle Cells

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Insulin increases glucose uptake into muscle by enhancing the surface recycling of GLUT4 transporters. In myoblasts, insulin signals bifurcate downstream of phosphatidylinositol 3-kinase into separate Akt and Rac/actin arms. Akt-mediated Rab-GAP activity (16, 17), reduces the duration of vesicle halting prior to fusion (17, 18), and elevates the efficiency of vesicle docking and fusion with the plasma membrane (16). Recent models debate as to which of these is regulated by insulin signals and which is rate-limiting. In adipocytes, there is contrasting evidence for the modulation by insulin of each of these steps. In an in vitro reconstitution assay, insulin enhanced the formation of GLUT4-containing vesicles (11). Insulin also increased the frequency of long range movements of GLUT4 vesicles without an accompanying change in their mean velocity (12–15). And recent studies by total internal reflection fluorescence microscopy (TIRFM) show that within 200 nm of the membrane, insulin promotes stalling of previously mobile GLUT4 vesicles (16, 17), reduces the duration of vesicle halting prior to fusion (17, 18), and elevates the efficiency of vesicle fusion with the cell surface (17, 19, 20). However, it is not known whether these events at the plasma membrane suffice to draw GLUT4 from perinuclear and cytosolic loci. Moreover, little is known about the regulation of GLUT4 traffic steps in muscle, yet skeletal muscle is the primary site of glucose disposal in vivo (21).

Insulin initiates a signaling cascade where activation of phosphatidylinositol 3-kinase (PI3K) is pivotal in achieving the gain in surface GLUT4 (22). We showed earlier that submembrane accrual and fusion of GLUT4 vesicles can be elicited by PI3K activators; PI3K and its phospholipid products (23). Downstream of PI3K, insulin signals bifurcate into at least two separate arms, both required for the net gain in membrane GLUT4. One arm...
involves Rac leading to actin remodeling (24); and perturbing actin dynamics largely diminishes insulin-stimulated GLUT4 traffic in muscle and adipose cells (25). The second arm involves activation of Akt. As there is no input of Akt on actin remodeling (26) nor of Rac on Akt activation (27); these represent parallel input signals required for GLUT4 traffic in muscle (28).

The Akt target AS160 is a Rab-GAP thought to be inactivated upon phosphorylation in response to insulin (29). This then allows signaling to proceed via its target Rabs, primarily Rab8A in muscle cells (28, 30) and Rab10 in adipocytes (31). Akt-mediated phosphorylation of AS160 participates in the insulin-dependent gain in surface GLUT4 in adipose (29) and muscle (32) cells in culture, and is impaired in skeletal muscle of type 2 diabetic patients (33). There is controversy as to the step in GLUT4 traffic impacted by AS160 in adipocytes, whether it is GLUT4 provision from the insulin-responsive pool (34) or interaction of GLUT4 vesicles with the plasma membrane (18, 20). This question has not been explored in muscle cells, and there is no information on the steps affected by the AS160 target Rabs or Rac and actin remodeling in either cell type.

The objective of this study was to discern the roles of the two signaling arms downstream of PI3K (Rac-dependent actin dynamics and Akt effectors AS160 and Rab8A), in distinct stages of GLUT4 traffic in L6 muscle cells.

EXPERIMENTAL PROCEDURES

Reagents and Constructs—Latrunculin B (LB), DMSO (high pressure liquid chromatography (HPLC) grade), and polyclonal anti-FLAG antibody were from Sigma-Aldrich. Hanks balanced salt solution was from Mediatech Inc. (Herndon, VA). Polyclonal (A-14) and monoclonal (9E10) anti-FLAG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA mAb was from Cell Signaling Technology Inc. (Danvers, MA). Anti-Rab8 mAb was from BD Biosciences (Mississauga, Ontario, Canada). Indocarbocyanine (Cy3), Cy5, Alexa488-bound secondary antibodies, and rhodamine-bound phallolidin were from Invitrogen, and horseradish peroxidase-bound goat secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Enhanced GFP-tagged K-Ras tail (K-Ras-GFP) cDNA (C-terminal K-Ras tail sequence (SKDGKKKKKKSKTKCVIM) as denoted (35)) was a kind gift of Dr. Mark Philips (Department of Medicine, New York University School of Medicine, New York) and Dr. Sergio Grinstein (Program in Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada). Dominant negative Rac1 tagged to the HA epitope (HA-DN-Rac) was from Dr. Alan Hall (MRC Laboratory for Molecular Cell Biology, London, UK). mAb to tetanus toxin light chain (TeTx) and TeTx cDNA were kind gifts from Dr. Gustav E. Lienhard (Dartmouth Medical School). Small inhibitory RNAs (siRNAs) targeted against Rab8A (siRab8A, CAG CGC GAA GGC CAA CAT CAA), Rab8B (siRab8B, AAC GAT AGA ACT CGA CCG AAA), and nonrelated controls (siNR; AAT AAG GCT ATG AAG AGA) were quickly chilled with ice-cold PBS + (with Ca$^{2+}$ and Mg$^{2+}$), and for 10 min each myoblasts were fixed with 4% paraformaldehyde, quenched with 0.1 M glycine, and blocked with 5% milk or goat serum before reacting with anti-myc antibody (1:100) for 1 h. To detect FLAG- or HA-tagged proteins, cells permeabilized with 0.1% Triton X-100 for 20 min were reacted with polyclonal anti-FLAG (1:1000) or monoclonal anti-HA (1:3000) antibodies, respectively, for 1 h. Cells were then stained 1 h with fluorescent Cy3-bound goat anti-mouse and Alexa488-bound goat anti-rabbit secondary antibodies (1:750) as noted.

To detect surface myc epitope in permeabilized cells, roundsed-up myoblasts were quickly chilled with ice-cold PBS+, fixed with 4% paraformaldehyde for 20 min, quenched with 0.1 M glycine for 10 min, permeabilized with 0.1% Triton X-100 for 20 min, blocked with 5% milk or goat serum for 10 min, and reacted for 1 h with primary antibodies (anti-myc were kind gifts from Dr. Gustav E. Lienhard (Dartmouth Medical School). Small inhibitory RNAs (siRNAs) targeted against Rab8A (siRab8A, CAG CGC GAA GGC CAA CAT CAA), Rab8B (siRab8B, AAC GAT AGA ACT CGA CCG AAA), and nonrelated controls (siNR; AAT AAG GCT ATG AAG AGA) were purchased from Qiagen (Mississauga, Ontario, Canada). All DNA constructs for transfections were prepared using Qiagen Hi-Speed Maxi-prep columns according to manufacturer’s protocol.

Cell Culture, Transfections, Insulin Treatment, and Immunoblotting—L6 cells stably expressing GLUT4myc (L6-GLUT4myc) were cultured and transfected with cDNAs using Lipofectamine 2000® (Invitrogen) as outlined previously (7, 36), or with siRNAs using the calcium phosphate-based CellPhect transfection kit (Amersham Biosciences) as per the manufacturer’s protocol. Unless stated otherwise, after 24 h for cDNA or 48 h for siRNA transfections, cells were serum-deprived for 4 h prior to stimulation with 100 nM insulin for 10 min at 37 °C. Equal protein siRNA-treated cell lysates, prepared and resolved by 13% SDS-PAGE prior to transferring to polyvinylidene difluoride as described previously (7, 36), were immunoblotted with anti-Rab8 (1:1000) mAb followed by horseradish peroxidase-bound goat anti-mouse (1:50,000) secondary antibody and detected by ECL.

Surface GLUT4myc and Submembrane Accumulation in Rounded-up Myoblasts—Rounded-up L6-GLUT4myc myoblasts, described earlier (23, 37, 38), afford greater resolution in differentiating surface-bound from intracellular epitopes. Adhered L6-GLUT4myc myoblasts were transfected with cDNAs (2 μg of FLAG-AS160-WT, FLAG-AS160-4P, HA-DN-Rac, or TeTx; 1 μg of K-Ras tail-GFP; or 0.6 μg of VAMP2-GFP) or siRNAs (100 nM siNR, siRab8A, or siRab8B alone; or 50 nM each siRab8A and siRab8B together) as noted. Serum-starved cells, detached from the substratum using PBS-free (without Ca$^{2+}$ or Mg$^{2+}$) for 20 min at 37 °C, were reattached to allow formation of rounded-up myoblasts. To disrupt actin, cells were treated with 250 nM LB in a 20-min PBS-free incubation. During replating onto glass cover slips, cells were treated with or without insulin and/or LB at 37 °C for 10 min as shown. Under control conditions corresponding to LB, DMSO was added to the same final amount.

To detect surface myc epitope, intact rounded-up myoblasts were quickly chilled with ice-cold PBS+ (with Ca$^{2+}$ and Mg$^{2+}$), and for 10 min each myoblasts were fixed with 4% paraformaldehyde, quenched with 0.1 M glycine, and blocked with 5% milk or goat serum before reacting with anti-myc antibody (1:100) for 1 h. To detect FLAG- or HA-tagged proteins, cells permeabilized with 0.1% Triton X-100 for 20 min were reacted with polyclonal anti-FLAG (1:1000) or monoclonal anti-HA (1:3000) antibodies, respectively, for 1 h. Cells were then stained 1 h with fluorescent Cy3-bound goat anti-mouse and Alexa488-bound goat anti-rabbit secondary antibodies (1:750) as noted.

To detect total myc epitope in permeabilized cells, rounded-up myoblasts were quickly chilled with ice-cold PBS+, fixed with 4% paraformaldehyde for 20 min, quenched with 0.1 M glycine for 10 min, permeabilized with 0.1% Triton X-100 for 20 min, blocked with 5% milk or goat serum for 10 min, and reacted for 1 h with primary antibodies (anti-myc
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antibody 1:100; polyclonal anti-FLAG antibody 1:1000; monoclonal anti-T-eTx 1:100 or anti-HA 1:3000 antibodies) as specified. Cells were stained 1 h with fluorescent Cy3-bound goat anti-mouse and Alexa488-bound goat anti-rabbit secondary antibodies (1:750) as shown.

After mounting onto glass slides with DakoCytomation mounting medium, cells were visualized by confocal microscopy using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired by scanning along the z axis every 1 mm using the ×63 objective at the same gain setting for all conditions. At least 15–20 fields selected at random were examined per condition, and all cells within each field were scored in order to collect at least 60 cells from at least 3–4 separate experiments.

To score surface fluorescent rims in intact cells, fluorescence intensity gains were set to avoid saturation in the insulin-stimulated state and to barely detect the external surface myc rim in unstimulated cells, in order to subsequently score the insulin-dependent gain in surface myc signal. The percentage of cells with labeled rims was scored and is an index of GLUT4myc epitope exposure in response to insulin. By this analysis, any clear rim of surface GLUT4myc was scored as positive, avoiding the ambiguity of detecting rims of different intensities. By analyzing numerous cells, it is expected that the behavior of a cell population is gauged. This approach to measuring the percentage of cells with rims has been repeatedly used in the GLUT4 literature, and is valid given the comparable expression of GLUT4myc in all cells of the L6-GLUT4myc clonal cell line (see Refs. 7 and 39). The number of myc signals per cell in a myoblast monolayer was 73 ± 5 (39). In the present study, the insulin response of gain in surface GLUT4myc was highly reproducible across experiments (Figs. 1B, 3, E, F, 6B, and 8B).

To score fluorescent rims of total myc and overlap with K-Ras tail-GFP in permeabilized cells, the proportion of cells with complete or partial rims (scored by quartiles) of peripheral GLUT4myc was determined separately, as well as the proportion of cells with rims that overlapped with the plasma membrane K-Ras tail-GFP signal. Rim coverage by quartile and overall colocalization with K-Ras tail-GFP were determined by examining each of the confocal planes of each cell. The single wavelength and “merge” images shown are from collapsed fields.

To further illustrate the localization of the total myc signal relative to the cell membrane K-Ras tail-GFP signal, images were acquired by scanning along the z axis every 0.2 μm using the ×63 objective and subjected to image deconvolution using the iterative restoration algorithm in the Velocity 3.0® software (Improvision Inc., Lexington, MA). Shown are single plane images following deconvolution.

Detection of Actin Filaments in Rounded-up Myoblasts—Adhered L6-GLUT4myc myoblasts were transfected with cDNA constructs (2 μg of FLAG-AS160-WT, FLAG-AS160-4P, HA-DN-Rac, or TeTx or 0.6 μg of VAMP2-GFP) or siRNAs (100 nM siNR or siRab8A) as shown. Detached serum-starved cells were replated to form rounded-up myoblasts and treated with or without insulin and/or LB as denoted above. Rounded-up myoblasts quickly chilled with ice-cold PBS+ were fixed with 4% paraformaldehyde, quenched with 0.1 M glycine, permeabilized with 0.1% Triton X-100 for 3 min, blocked with 5% milk, and stained for 1 h with primary anti-FLAG (1:1000) or anti-HA (1:3000) antibodies followed by fluorescence-conjugated Alexa488-bound goat anti-rabbit or anti-mouse secondary antibodies (1:750) in the presence of rhodamine-conjugated phallacidin (1.1 μl/ml) to label F-actin. Cells were mounted and visualized by confocal microscopy as described above. Images were acquired by scanning along the z axis every 1 μm using the ×63 objective at the same gain setting for all conditions. At least 10–15 fields were examined to collect 50 cells/condition from at least three separate experiments.

Electron Microscopy—L6-GLUT4myc myoblasts grown on glass coverslips were serum-starved and treated with 100 nM insulin for 10 min. Cells were then extracted with 0.75% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, 1 μM phallacidin, pH 6.9) with protease inhibitors (leupeptin, aprotinin, and benzamidine at 10 μg/ml each) for 2 min at 37 °C.

To label GLUT4myc with colloidal gold, the above extracted cells (shells) were washed with PHEM buffer followed by a 10-min fixation with 0.1% glutaraldehyde at 37 °C and 2 min of quenching with 1 mg/ml sodium borohydride at room temperature. Shells were washed with Hanks balanced salt solution, blocked, and labeled with primary anti-myc mAb (1:10 in 1% bovine serum albumin) overnight and then with secondary antibody (1:15, goat anti-mouse IgG conjugated to 10-nm colloidal gold particles) for 2 h. Shells were washed extensively, fixed with 1% glutaraldehyde for 10 min, and washed with double distilled water. Coverslips were rapidly frozen on a helium-cooled copper block, freeze-dried at −85 °C in a Cressington freeze-fracture machine (CFE-50; Ratford, Watford, UK), and rotary-coated with 1.2 nm of platinum at a 45° angle followed by coating with 4 nm of carbon at 90° without rotation. Replicas were floated off of the coverslips by using 25% hydrofluoric acid, washed in water, and collected on a 200-mesh copper grids coated with Formvar. Digital images of the replicas (50–200 nm magnification) were collected using a FEI Tecnai 20 transmission electron microscope at an 80-kV accelerating voltage.

To label actin filaments with myosin S1 (40) after the extraction step, shells were washed with PHEM buffer, decorated with 2 mg/ml myosin S1 for 15 min at room temperature, washed extensively, and then fixed for 10 min with 1% glutaraldehyde and 0.1% tannic acid in 10 mM sodium phosphate buffer (pH 7.5) at room temperature. Samples were washed with double distilled water and frozen rapidly as described above. Coverslips were rotary coated with 2 nm of platinum at a 45° angle followed by coating with 5 nm of carbon at 90° without rotation, and replicas were prepared for image collection as described above.

Statistical Analysis—Statistical analyses were carried out using Prism 3.0 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.
statistical analysis. We used an L6 clonal muscle cell line that

were transfected with or without 0.6 μg of toxin-sensitive
VAMP2-GFP (V2) or 2 μg of TeTx CDNAs were replated with or without 100 nM insulin for 10 min and processed intact for surface GLUT4myc or permeabilized to detect actin by immunofluorescence. A, representative images show surface GLUT4myc rims. Detached serum-starved L6-GLUT4myc myoblasts transfected with or without insulin in intact cells transfected with empty vector control (CON) (a, d), V2 (b, e), or V2/TeTx (c, f). B, the proportion of cells assaying the insulin-induced surface GLUT4myc response above basal in control, V2, or V2/TeTx cells is quantified as mean values ± S.E. based on ~60 cells/condition from three separate experiments. *, p < 0.05 relative to control. C, representative images show actin with or without insulin in control (a, d) or V2 (b, e) or V2/TeTx (c, f)-expressing cells. Size-reduced pseudo-colored insets highlight intact V2 (b, e) or cleaved V2 in cells with TeTx (c, f). Bars, 10 μm.

RESULTS

Preventing GLUT4myc Vesicle-Membrane Fusion Does Not Preclude Its Cortical Accumulation—A primary question of this study was whether insulin-derived signals affect specific steps in GLUT4 traffic in muscle cells, namely redistribution to the cell cortex or docking/fusion with the membrane. Accordingly, it was important to establish a reporter of TeTx action, because upon cleavage by the toxin VAMP2-GFP assumes a tight perinuclear localization in the Golgi apparatus that contrasts with its more disperse, natural distribution (7). To quantify the insulin response of GLUT4myc, the acquisition gain was set to see a minimal basal state signal, and the number of cells with insulin-induced rims was scored over several independent experiments (see “Experimental Procedures”). Under these scoring conditions, 94 ± 8% of control and 91 ± 5% of VAMP2-GFP-expressing cells showed insulin-dependent exofacial GLUT4myc rims. In contrast, only 9 ± 0% of TeTx-expressing cells displayed such insulin-mediated rims (Fig. 1B). TeTx expression did not affect formation of insulin-induced cortical actin projections (Fig. 1C).

Under such conditions of blocked GLUT4myc vesicle-membrane fusion, we analyzed the distribution of intracellular GLUT4myc following cell permeabilization. Myoblasts were transfected with K-Ras tail-GFP (K-Ras-GFP) as a plasma membrane marker (35, 43) to determine the localization of GLUT4myc vis-à-vis the cell periphery. Notably, the majority (95 ± 1%) of TeTx-expressing cells exhibited full cortical GLUT4myc rims in response to insulin, akin to insulin-treated control cells (96 ± 5%; Fig. 2, A and B). However, GLUT4myc accumulation at the cell cortex of insulin-stimulated TeTx-transfected cells minimally overlapped with the surface marker K-Ras-GFP (in only 12 ± 5% of these cells; Fig. 2B). Fig. 2C illustrates this submembrane GLUT4myc disposition in a TeTx-positive cell after insulin stimulation, revealed by image to deconvolution. Thus, TeTx-mediated blockage of GLUT4myc fusion with the plasma membrane per se did not prevent its cortical (submembrane) build-up in response to insulin.

Actin Dynamics Is Required for Peripheral Accumulation of GLUT4myc—Insulin signaling downstream of PI3K diverges into two discrete arms that together bring about the insulin-dependent gain in surface GLUT4. One arm engages actin dynamics, including Rac-dependent actin remodeling. Previous studies have implicated the actin cytoskeleton and Rac activity in the overall process of insulin-responsive GLUT4myc traffic in L6 cells (44, 45). Disrupting actin dynamics could have abrogated the mobilization of GLUT4 vesicles to the cell surface, their retention at the cell cortex, or their fusion with the plasma membrane.

To discern between these possibilities, L6 myoblasts were pretreated with 250 nM LB, or they were transiently transfected with a dominant negative Rac (DN–Rac) mutant that prevents actin remodeling (24). In unstimulated cells, actin filaments were detectable as a cortical band about the cell perimeter (Fig. 3, A and D). Consistent with prior findings (46), stimulation with insulin resulted in massive cortical actin projections at the cell surface. LB treatment (Fig. 3A) or DN-Rac expression (Fig.
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Complete GLUT4myc rims were detectable in 96 ± 6% of control insulin-stimulated cells but only in 13 ± 9% of LB-treated insulin-stimulated cells (Fig. 4, A and B). Likewise, cortical rims were evident only in a few of the DN-Rac-transfected cells (8 ± 4%) after insulin treatment, in contrast to the robust response of control cells (89 ± 2%; Fig. 5, A and B).

The merged images in Figs. 4A and 5A highlight the coincidence of Cy3-labeled GLUT4myc with plasma membrane-associated K-Ras-GFP in insulin-treated cells. Figs. 4B and 5B depict the proportion of cells with full GLUT4myc rims under the diverse treatments and of rims overlapping with K-Ras-GFP. There was virtually no GLUT4myc coincidence with K-Ras-GFP in LB-treated cells (only in 3 ± 4% of cells) compared with marked overlap in 92 ± 1% of control cells (Fig. 4B). Accordingly, there was no coincidence of GLUT4myc with K-Ras-GFP in DN-Rac cells (6 ± 3%) relative to 83 ± 3% in control cells (Fig. 5B).

Figs. 4C and 5C further emphasize the lack of cortical GLUT4myc in response in deconvolution images of LB-treated or DN-Rac-expressing cells, respectively. In comparison with Fig. 2 with TeTx, these findings suggest that the significant decrease in peripheral GLUT4myc accumulation seen upon actin disruption is unlikely to result from blocking vesicle fusion with the cell membrane.

AS160-4P Prevents GLUT4 Membrane Insertion and Reduces Its Cortical Accumulation—In addition to Rac/actin, Akt signals leading to Rab-GAP AS160 phosphorylation provides parallel input(s) to elicit the full recruitment of surface transporters by insulin. We recently showed that transient expression of the AS160-4P mutant diminished the insulin-induced surface GLUT4myc gain in monolayers of L6-GLUT4myc myoblasts (32). As shown in Fig. 6A, insulin caused its expected surface exposure of GLUT4myc in control or wild type AS160 (AS160-WT)-expressing cells. In contrast, the AS160-4P mutant virtually abolished myc epitope externalization by insulin. Only 19 ± 10% of cells expressing AS160-4P showed this insulin response compared with 97 ± 4% of AS160-WT-expressing and 99 ± 3% of control cells (Fig. 6B).

In permeabilized rounded-up L6 myoblasts, complete GLUT4myc peripheral rims were found in 94 ± 0% of control

3D) prevented this cortical actin remodeling. Supplemental Fig. S1A depicts the branched nature of the insulin-dependent cortical actin mesh upon EM analysis of platinum-shadowed membrane shells. Actin filaments appear “braided”; their direction is identified by the arrow-like projection of the S1 myosin decorations. Supplemental Fig. S1B shows the close proximity of GLUT4myc to filaments (likely actin) analyzed by EM.

Neither LB (Fig. 3B) nor DN-Rac (Fig. 3E) altered basal state surface GLUT4myc, but each largely reduced its insulin-induced membrane gain relative to control cells. As quantified in Fig. 3C, only 17 ± 4% of the cells treated with LB displayed an insulin-dependent increase in surface-exposed GLUT4myc compared with 91 ± 2% of control cells. Similarly, 16 ± 4% of DN-Rac-expressing cells had elevated surface GLUT4myc by insulin relative to 76 ± 3% of control cells (Fig. 3F).

In parallel, rounded myoblasts were permeabilized and the myc-epitope was detected vis-à-vis the K-Ras-GFP signal. Cells with complete GLUT4myc rims in the periphery were scored, as well as those where the rims colocalized with K-Ras-GFP.

**FIGURE 2.** Tetanus toxin does not alter the peripheral gain of GLUT4myc beneath the plasma membrane by insulin. Detached, serum-starved L6-GLUT4myc myoblasts transfected with 1 µg of K-Ras-GFP cDNA and 2 µg of empty vector (CON, control) or TeTx cDNAs were replated with or without 100 nM insulin for 10 min and permeabilized to detect total GLUT4myc and TeTx protein by immunofluorescence. A, representative images show peripheral GLUT4myc rims (total myc) with or without insulin in rounded myoblasts with K-Ras-GFP and control (rows a and c) or TeTx (rows b and d) cDNAs. Merged images for each condition are composites of K-Ras-GFP and total myc, where yellow pixels indicate areas of coincidence of green K-Ras pixels and red peripheral GLUT4myc pixels. Insets (in b and d) are size-reduced pseudo-colored images of TeTx protein expression in the same cells (reacted with anti-TeTx antibodies). Bars, 10 µm. B, the proportion of cells with complete peripheral GLUT4myc rims and of such rims overlapping with K-Ras is quantified on the left (black bars) and right (gray bars) axes, respectively. Values are means ± S.E. based on 60 cells/condition from three separate experiments. p < 0.05 relative to insulin, K-Ras. C, representative image of a cell transfected with K-Ras-GFP and TeTx after insulin was subjected to deconvolution. Shown are: a, peripheral GLUT4myc staining in red along with cell membrane K-Ras-GFP in green, where the inset is size-reduced pseudo-colored TeTx protein expression in the same cell (Bars, 10 µm); b, a region about the plasma membrane denoted by the white rectangle. Bar, 1 µm.
and 84 ± 5% of AS160-WT-transfected cells but in only 36 ± 14% of AS160-4P-transfected cells (Fig. 7, A and B, and supplemental Fig. S2A). Representative images show that AS160-4P precluded GLUT4myc colocalization with K-Ras-GFP evident in 85 ± 3% of control cells, albeit allowing submembrane build-up of the transporter. AS160-WT expression only slightly affected this response (74 ± 4% of cells had a full GLUT4myc rim overlapping with K-Ras-GFP). In contrast, virtually none (2 ± 3%) of the AS160-4P-expressing cells accrued this overlap. Hence, in the 36 ± 14% of AS160-4P-expressing cells showing peripheral GLUT4myc, the transporter is not inserted in the membrane but is instead accumulated in a cortical, submembrane zone. In this latter case, the full cortical GLUT4myc rim (>75–100%) failed to coincide with the surface marker, K-Ras-GFP, which is further underscored by the illustrated images of Fig. 7C, subjected to deconvolution.

The insulin-stimulated cells not displaying a complete peripheral GLUT4myc rim were further subdivided into those with partial rims covering more than half (50–75%) or covering none to less than half (0–50%) of the periphery (supplemental Fig. S2). AS160-4P augmented the proportion of cells with partial rims covering more than half of the periphery to 48 ± 10% (relative to 6 ± 0% in control cells or 14 ± 6% in AS160-WT-transfected cells, all insulin-treated). The proportion of cells with GLUT4myc covering none to less than half of the periphery also rose to 16 ± 11% (compared with negligible detection of this phenotype in control or AS160-WT-expressing cells).

Together, Figs. 6 and 7 and supplemental Fig. S2 suggest that significant amounts of GLUT4myc collect at the cell cortex of AS160-4P-expressing insulin-stimulated cells but are impeded from inserting into the membrane. Further, the partial formation of full submembrane rims of GLUT4myc (36 ± 14% versus the amply prevailing cortical rims in 95 ± 1% of TeTx-transfected cells, Fig. 2B) suggests that, independently of preventing GLUT4myc vesicle fusion, AS160-4P reduces the accumulation of the transporter beneath the membrane.

Effect of siRab8A on Surface Gain and Cortical Build-up of GLUT4myc—The Akt target AS160 has the signature of a Rab-GAP, and current models propose that its GAP activity is inactivated by insulin signaling via Akt (29) thereby allowing downstream signaling through its target Rabs. We recently showed that overexpressing WT or constitutively active Rab8A overcame the inhibitory effects of AS160-4P on surface GLUT4myc levels in L6 myoblasts (30). This rescuing effect was also seen with WT Rab14 but not with WT or constitutively active Rab10 or Rab14 (30).

To date, the step(s) of GLUT4 vesicle exocytosis regulated by any of these Rabs are still unknown. Here we have focused on Rab8A by exploring the effect on GLUT4 traffic of silencing this gene, using a selective small inhibitory RNA to this transcript (siRab8A).

Rab8A knockdown (Fig. 8C, see legend) reduced insulin-dependent surface GLUT4myc externalization in rounded-up myoblasts from 69 ± 1% observed in siNR-transfected cells to 22 ± 3% in siRab8A-transfected cells (Fig. 8B). In contrast, siRab8B did not prevent GLUT4 externalization, and joint transfection of siRab8A and siRab8B had no further action than siRab8A alone (data not shown).

In permeabilized cells, there was a marked loss of full peripheral GLUT4myc rims in siRab8A-transfected cells (7 ± 5%) compared with the observed cortical rims in 79 ± 5% of siNR-
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FIGURE 4. Latrunculin B markedly prevents insulin-dependent gain in submembrane GLUT4myc.
Detached, serum-starved L6-GLUT4myc myoblasts transfected with 1 μg of K-Ras-GFP cDNA and left untreated (CON) or pretreated with 250 nM LB for 20 min were replated with or without LB ± 100 nM insulin for 10 min and then permeabilized to detect total GLUT4myc by immunofluorescence. A, representative images show peripheral GLUT4myc rims (total myc) with or without insulin in rounded myoblasts transfected with K-Ras-GFP and left untreated as controls (CON) (rows a and c) or pretreated with LB (rows b and d). Merged images for each condition are composites of K-Ras-GFP and total myc, where yellow pixels indicate areas of coincidence of green K-Ras pixels and red peripheral GLUT4myc pixels. Bar, 10 μm. B, the proportion of cells with complete peripheral GLUT4myc rims and of such rims overlapping with K-Ras is quantified on the left (black bars) and right (gray bars) axes, respectively. Values are means ± S.E. based on ~60 cells/condition from three separate experiments. *p < 0.05; #p < 0.05 relative to insulin, K-Ras, respectively. C, representative image of a cell transfected with K-Ras-GFP and total myc, where yellow pixels indicate areas of coincidence of green K-Ras pixels and red peripheral GLUT4myc pixels. Bar, 10 μm.

DISCUSSION

Characteristics of GLUT4 Traffic in Muscle Cells—Skeletal muscle is the main site of glucose disposal in response to insulin in vivo, and this response is vastly attenuated in insulin-resistant states, notably type 2 diabetes. Hence, it is of paramount importance to understand the molecular mechanisms underlying insulin-dependent glucose uptake, which occurs through GLUT4. The L6 muscle cell line stably expressing myc-tagged GLUT4 offers the possibility of examining GLUT4 traffic in response to physiological stimuli and conditions that elicit insulin resistance (36, 49). In L6 myoblasts, as in myotubes, half of the GLUT4 segregates away from other recycling proteins (data not shown; Ref. 50), continuously cycles to the cell surface, and displays insulin-dependent acceleration of its exocytic traffic through vesicles that rely on VAMP2 for fusion with the membrane (7). Moreover, the magnitude of insulin-dependent (most cells remained in the 0–25% quartile of GLUT4myc peripheral coverage), resembling control unstimulated cells. In contrast, siRab8A allowed the formation of insulin-dependent rims that covered ≥25–50% of the periphery in about half of the cells. These results suggest that Rac and Rab8A may have different contributions to the steps in GLUT4 traffic (see “Discussion”).

AS160-4P and Rab Knockdown Do Not Impinge on Actin Remodeling—As Rab8A can exert a regulatory input on the cytoskeleton in other cell types (47, 48), we examined whether Rab8A knockdown might disrupt insulin-induced actin remodeling and in this way contribute to the ineffective accumulation of GLUT4myc at the cell cortex. In contrast to the actin-disrupting agents (Fig. 1), siRab8A transfection did not alter actin filament organization in the unstimulated (seen as a cortical band) or insulin-stimulated (seen as cortical projections) states (supplemental Fig. S3A). Similarly, neither AS160-WT nor AS160-4P altered actin filament appearance in unstimulated or insulin-stimulated cells (supplemental Fig. S3B). Interestingly, AS160-WT and AS160-4P localized to regions coinciding with the insulin-remodeled actin mesh (supplemental Fig. S3B), suggesting that the effective action of AS160 on GLUT4 vesicle traffic may occur at the cell cortex.
gain in surface GLUT4 is compatible with the stimulation of glucose uptake in isolated skeletal muscles.

Approaches to Understanding the Regulation of GLUT4 Traffic—Recent effort has been devoted to identifying which and how many steps are targets of regulation by insulin in fat cells. Much less is known about the events involved in GLUT4 traffic in muscle cells and their susceptibility to regulation. This becomes ever more relevant as recent findings suggest that insulin signals required for GLUT4 transloca-

tion in muscle cells bifurcate downstream of PI3K; Rac signals, through actin remodeling, and Akt signals, through AS160 and its target Rab(s), are required independently for GLUT4 traffic (27, 28, 32). The aim of this study was to identify which states in GLUT4 traffic are targets of regulation by these separate arms of insulin signaling. We took advantage of the resolution of confocal fluorescence microscopy applied to rounded-up myoblasts and the availability of agents that arrest vesicle fusion (TeTx) or demarcate the cell surface (K-Ras-GFP). We applied these probes and strategy to analyze GLUT4myc localization in cells with actin dynamics interrupted by LB or DN-Rac and in cells expressing a mutant AS160, which cannot respond to Akt, or siRNA that selectively knocks down Rab8A. The quantitative analysis of GLUT4myc fluorescence in fixed cells allowed us to define its localization to the perinuclear, submembrane, or cortical area (fluorescence within 1 μm of the membrane but without colocalization with K-Ras-GFP) and the plasma membrane proper. The latter was defined both by exposure of the myc epitope to the extracellular space and by colocalization with K-Ras-GFP.

Actin Dynamics, AS160, and Rab8A Regulate Distinct Events in GLUT4 Traffic—Treatment of myoblasts with LB or transient expression of a DN-Rac mutant, which prevented insulin-induced actin remodeling in rounded-up myoblasts (Fig. 3), precluded GLUT4myc accumulation in the cortical area, and accordingly the transporter did not colocalize with the surface marker, K-Ras-GFP (Figs. 4 and 5). Consistent with this behavior, there was no gain in exofacially exposed GLUT4myc on disrupting actin dynamics (Fig. 3). The reduced number of cells with full GLUT4myc rims is not deemed to result from the abrogated fusion, as most cells expressing TeTx showed complete rims of submembrane GLUT4myc without any appreciable GLUT4myc fusion with the membrane (Figs. 1 and 2).
Rac, AS160, and Rab8A Distinctly Regulate GLUT4 Traffic

FIGURE 7. AS160-4P partly reduces insulin-dependent submembrane accumulation of GLUT4myc. Detached, serum-starved L6-GLUT4myc myoblasts transfected with 1 μg of K-Ras-GFP cDNA and 2 μg of empty vector (CON, control), FLAG-AS160-WT, or FLAG-AS160-4P cDNAs were replated with 100 nM insulin for 10 min and permeabilized to detect total GLUT4myc and FLAG-tagged AS160 proteins by immunofluorescence. A, representative images show peripheral GLUT4myc rims (total myc) with or without insulin in rounded myoblasts transfected with K-Ras-GFP along with empty vector control (rows a and d), AS160-WT (rows b and e), or AS160-4P (rows c and f) cDNAs. Merged images for each condition are composites of K-Ras-GFP and total myc, where yellow pixels indicate areas of coincidence of green K-Ras pixels and red peripheral GLUT4myc pixels. Insets (in b, c, e, and f) are respective size-reduced pseudo-colored FLAG-tagged AS160 proteins expressed in the same cells (reacted with anti-FLAG antibodies). Bars, 10 μm. There was no appreciable difference in AS160-WT and AS160-4P distribution comparing numerous images with similar expression levels of these proteins. B, the proportion of cells with complete peripheral GLUT4myc rims above basal level and of such rims overlapping with K-Ras is quantitated on the left (black bars) and right (gray bars) axes, respectively. Values are means ± S.E. based on ∼60 cells/condition from three separate experiments. *, p < 0.05; #, p < 0.05 relative to insulin, K-Ras, respectively. C, a representative image of a cell transfected with K-Ras-GFP and AS160-4P with a full insulin-induced peripheral GLUT4myc rim was subjected to deconvolution. Show are: a, peripheral GLUT4myc staining in red with cell membrane K-Ras-GFP in green, where the inset is size-reduced pseudo-colored FLAG-AS160-4P protein expression in the same cell (Bars, 10 μm); b, a region about the plasma membrane denoted by a white rectangle. Bar, 1 μm.

Hence, abrogation of vesicle fusion with the plasma membrane does not in itself suffice to cause a “back-up” of GLUT4 such that its build-up beneath the membrane would be diminished. The lack of cortical amassing of GLUT4myc caused by LB or DN-Rac expression suggests that actin filament dynamics is involved in either vesicle mobilization toward the cell membrane or in its retention at the cortex. Supporting the latter scenario is the observation that insulin-induced actin remodeling occurs toward the cell periphery (cortical actin remodeling, Fig. 3) and that, by EM, the majority of cortical GLUT4myc molecules lies in close proximity to cytoskeletal filaments in insulin-stimulated cells (supplemental Fig. S1).

AS160-4P, which cannot respond to insulin because of mutations of sites normally phosphorylated by Akt, abrogated the gain in exofacially exposed GLUT4myc (Fig. 6). Under these conditions, however, 36% of the cells still displayed full cortical rings of GLUT4myc that did not colocalize with K-Ras-GFP (Fig. 7). Another 48% of the cells displayed partial submembrane rings of GLUT4myc that also did not overlap with K-Ras-GFP (supplemental Fig. S2). Together, these results suggest that insulin-responsive AS160 is required for GLUT4myc fusion with the membrane. They also suggest that, in addition, insulin-responsive AS160 contributes to the effective build-up in cortical GLUT4myc (see below). These two inputs may reconcile the apparently discordant reports on the action of AS160 in 3T3-L1 adipocytes, which propose that the regulation of this protein impacts on GLUT4 docking/fusion with the plasma membrane (18, 20, 51) or its exit from dynamic storage pools (34, 52). Along with the observations discussed above, this result establishes the fact that the different events in GLUT4myc traffic can be discerned by confocal fluorescence microscopy of permeabilized rounded-up myoblasts, such that full, partial, or no accumulation of GLUT4myc in the cortical zone can be quantified.

The two inputs by AS160 on vesicle docking/fusion and cortical vesicle accumulation may result from regulation through different targets of AS160. Indeed, the Tre-2/Bub2/Cdc16 (TBC) domain of AS160 inactivates Rabs 2A, 8A, 10, and 14. In 3T3-L1 adipocytes, knockdown of Rab10 expression prevented the insulin-dependent gain in surface GLUT4 (31), and a recent report assigns to Rab10 a role in the recycling of neuronal cargo internalized via cholesterol-dependent endocytosis (53). GLUT4 internalization through this route is more absolute in adipocytes (54) than in muscle cells (55). Using a gain of function approach in L6 muscle cells, we achieved significant restoration of GLUT4myc membrane exposure by overexpression of Rabs 8A and 14 but not Rab10 in cells expressing AS160-4P (30). A caveat of this approach is that overexpressing Rabs may sequester and thus override the inhibitory effects of AS160-4P. Using the alternate strategy of gene silencing, Rab8A knockdown (but not Rab10 knockdown; data not shown) prevented the insulin-induced surface GLUT4myc gain (Fig. 8A). This allowed us to examine the intracellular localization of the transporter under these conditions. Interestingly, most siRab8A-treated cells lacked a complete submembrane GLUT4myc rim, contrasting with the effect of TeTx (Fig. 2). Hence, Rab8A knockdown appears to interfere with GLUT4 traffic at a stage prior to docking/fusion with the membrane. However, half of the cells with silenced Rab8A showed some level of submembrane accumulation of GLUT4myc (>25–50% coverage, Fig. 10). This differs from GLUT4myc localization in cells where actin dynamics is prevented via DN-Rac (as most cells lacked any appreciable level of submembrane GLUT4myc). We speculate that Rab8A silencing may limit exit of GLUT4 vesicles from the dynamic retention cycle, but those escaping can be retained at the cortex. Of interest, Rab8A regulates traffic of melanosomes to the cell periphery, AMPA receptors to the synaptic membrane, and trans-Golgi network (TGN)-derived vesicles to the basolateral plasma membrane in Madin-Darby canine kidney cells (MDCK) cells (56–58).

Dynamic Models of GLUT4 Traffic—A current model of GLUT4 traffic in adipose cells proposes continuous GLUT4
FIGURE 8. Selective siRNA-mediated Rab8A knockdown prevents gain in surface GLUT4 myc.
L6-GLUT4myc myoblasts were transfected with 100 nM siNR, siRab8A, or siRab8B or, when together, siRab8A and siRab8B at 50 nM each as noted. A, detached, serum-starved cells were replated ± 100 nM insulin for 10 min and processed intact for surface GLUT4-my by immunofluorescence. Representative images show surface GLUT4myc rims with or without insulin in intact cells transfected with siNR (a and c) or siRab8A (b and d). Bar, 10 μm. B, the proportion of cells amassing the insulin-induced surface GLUT4myc response is quantified in siNR- or siRab8A-transfected cells. Values are means ± S.E. based on ~60 cells/condition from three separate experiments. *, p < 0.05 relative to siNR. C, equal protein lysates from siRNA-treated cells were immunoblotted with anti-Rab8 antibody, which detects Rab8A and Rab8B. Shown is a representative blot of immunodetected Rab8 proteins from four separate experiments. As seen, siRab8A transfection selectively reduced proteins detected by anti-Rab8 antibody to 53 ± 2% (*, p < 0.05 relative to siNR). Combined action of siRab8A and siRab8B caused virtual loss of immunodetectable Rab8 proteins, suggesting that each siRNA was markedly effective in knocking down its cognate Rab8 isoform.

FIGURE 9. Rab8A knockdown prevents insulin-induced cortical GLUT4myc build-up. Detached, serum-starved L6-GLUT4myc myoblasts transfected with 1 μg of K-Ras-GFP cDNA and 100 nM siNR or siRab8A were replated ± 100 nM insulin for 10 min and permeabilized to detect total GLUT4myc by immunofluorescence. A, representative images show peripheral GLUT4myc rims (total myc) with or without insulin in rounded myoblasts transfected with K-Ras-GFP along with siNR (rows a and c) or siRab8A (rows b and d). Merged images for each condition are composites of K-Ras-GFP and total myc, where yellow pixels indicate areas of coincidence of green K-Ras pixels and red peripheral GLUT4myc pixels. Bar, 10 μm. B, the proportion of cells with complete peripheral GLUT4myc rims and the proportion of such rims overlapping with K-Ras are quantified on the left (black bars) and right (gray bars) axes, respectively. Values are means ± S.E. based on ~80 cells/condition from four separate experiments. *, p < 0.05; #, p < 0.05 relative to insulin, K-Ras siNR, respectively.
such as α-actinin4 (60), the tether may possibly involve exocyst components (64, 65). However, one must also consider the prospect that AS160, via Rab8A, contributes to making GLUT4 vesicles more available at the cell surface, perhaps by relieving the idle cycle of GLUT4 between the specialized pool and recycling endosome (1). Indeed, a second model proposes that insulin increases the number of GLUT4 vesicle movement events toward the membrane (15); this is not exclusive of further insulin signal input at the levels of tethering, docking, and fusion. Analyzing our results by this model, AS160-4P would taper the net gain in GLUT4 vesicles near the periphery, so that on top of arresting GLUT4 vesicle docking/fusion, the cortical build-up of GLUT4 would be less effective (producing, at steady state, at least in the time period studied, fewer cells with full peripheral rims). Rab8A may potentially act downstream of AS160 phosphorylation (which is presumed to prevent Rab inactivation) to promote GLUT4 release from its dynamic retention, making it available for transit to the periphery. One can hypothesize that this occurs through alterations in fusion/fission events within endosomes, not unlike the control of cargo recycling exerted by Rabs 4 and 11 and their interacting proteins (66). Future studies of GLUT4 vesicle dynamics in real time will be required to discern between these possibilities. Such studies will also reveal whether AS160-4P prevents GLUT4 vesicle fusion (as suggested by its effect on insulin action at 10 min) or only delays it.

Finally, AS160-4P distinctly abrogates GLUT4myc membrane insertion; this may reflect a regulatory input at the level of the vesicle-membrane fusion machinery. Based on the selective requirement of SNARE proteins (VAMP2, syntaxin4, and SNAP23 regulated by Munc18c) for insulin-dependent GLUT4 vesicle-membrane fusion (67), the chance that AS160 impinges on SNARE complex formation warrants future examination. Although this requires direct experimental analysis, one may hypothesize that: (i) Rab targets of AS160 (e.g. Rab8A) might regulate the acquisition of VAMP2 during vesicle release; (ii) other Rab targets of AS160 might regulate syntaxin, akin to the role of Rab3 in synaptic vesicle fusion (68, 69); (iii) 14-3-3 proteins that bind to AS160 in a regulated fashion (70) might impinge on Munc18c or SNARE proteins that are regulated by phosphorylation.

In conclusion, we propose a working model whereby actin dynamics, AS160, and Rab8A impact on identifiable stages in insulin-regulated GLUT4 traffic (supplemental Fig. S4). According to this hypothetical model, dynamic actin filaments are essential for cortical GLUT4 retention, likely via tethering mechanisms at the cell cortex (although we cannot currently rule out participation of vectorial mobilization toward the membrane). Conversely, AS160 is essential for docking/membrane insertion of GLUT4, and whether this action requires a Rab protein will be the subject of future investigation. AS160 also contributes to GLUT4 availability in close proximity to the membrane, a process that might be mediated by Rab8A. Ultimately, these findings could direct the search for strategies to alleviate insulin resistance by targeting individual steps of GLUT4 traffic.

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