Myo1c binding to submembrane actin mediates insulin-induced tethering of GLUT4 vesicles

Shlomit Boguslavsky, Tim Chiu, Kevin P. Foley, Cesar Osorio-Fuentealba, Costin N. Antonescu, K. Ulrich Bayer, Philip J. Bilan, and Amira Klip

Cell Biology Program, Hospital for Sick Children, Toronto, ON M5G 1X8, Canada; FONDAP-CEMC Instituto de Ciencias Biomedicas, University of Chile, Santiago 6530499, Chile; Department of Chemistry and Biology, Ryerson University, Toronto, ON MSB 2K3, Canada; Department of Pharmacology, University of Colorado Denver–School of Medicine, Aurora, CO 80045-0508

ABSTRACT GLUT4-containing vesicles cycle between the plasma membrane and intracellular compartments. Insulin promotes GLUT4 exocytosis by regulating GLUT4 vesicle arrival at the cell periphery and its subsequent tethering, docking, and fusion with the plasma membrane. The molecular machinery involved in GLUT4 vesicle tethering is unknown. We show here that Myo1c, an actin-based motor protein that associates with membranes and actin filaments, is required for insulin-induced vesicle tethering in muscle cells. Myo1c was found to associate with both mobile and tethered GLUT4 vesicles and to be required for vesicle capture in the total internal reflection fluorescence (TIRF) zone beneath the plasma membrane. Myo1c knockdown or overexpression of an actin binding–deficient Myo1c mutant abolished insulin-induced vesicle immobilization, increased GLUT4 vesicle velocity in the TIRF zone, and prevented their externalization. Conversely, Myo1c overexpression immobilized GLUT4 vesicles in the TIRF zone and promoted insulin-induced GLUT4 exposure to the extracellular milieu. Myo1c also contributed to insulin-dependent actin filament remodeling. Thus we propose that interaction of vesicular Myo1c with cortical actin filaments is required for insulin-mediated tethering of GLUT4 vesicles and for efficient GLUT4 surface delivery in muscle cells.

INTRODUCTION Actin filaments are fundamental elements in intracellular vesicle traffic (Ridley, 2006; Lanzetti, 2007), in part through their continuous assembly and disassembly (i.e., actin remodeling; Blanchion et al., 2000; Pollard, 2007). Actin dynamics is required for budding, fission, and transport of Golgi carriers (Egea et al., 2006), and cortical actin remodeling beneath the plasma membrane is required for efficient endocytic and exocytic events at the plasma membrane (Lanzetti, 2007). Actin filament dynamics is also notably involved in the insulin-dependent translocation of glucose transporter GLUT4 to the membranes of muscle and fat cells (Tsakiridis et al., 1994; Kanzaki and Pessin, 2001; Tong et al., 2001; Lopez et al., 2009; Chiu et al., 2011b; Stockli et al., 2012). Insulin-dependent GLUT4 translocation is the rate-limiting step in the capture of glucose, a phenomenon particularly important in skeletal muscle, as this is the primary tissue responsible for dietary glucose disposal.

GLUT4-containing vesicles continuously cycle between the plasma membrane and several intracellular compartments (e.g., endosomes, trans-Golgi network), and insulin promotes GLUT4 vesicle exocytosis (Dugani and Klip, 2005). Extensive research shows that insulin-induced intracellular signals regulate the arrival, tethering, docking, and fusion of GLUT4 vesicles with the membrane (reviewed by Foley et al., 2011; Stockli et al., 2012). Whereas docking and fusion are mediated by a subset of soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins (Foster and Klip, 2000; Watson and Pessin, 2007; Zaid et al., 2008; Stockli et al., 2012), GLUT4 exocytosis requires the rapid arrival of vesicles to the plasma membrane (Kanzaki and Pessin, 2001; Tong et al., 2001; Lopez et al., 2009; Chiu et al., 2011b). Myo1c was found to associate with both mobile and tethered GLUT4 vesicles and to be required for vesicle capture in the TIRF zone beneath the plasma membrane. Myo1c knockdown or overexpression of an actin binding–deficient Myo1c mutant abolished insulin-induced vesicle immobilization, increased GLUT4 vesicle velocity in the TIRF zone, and prevented their externalization. Conversely, Myo1c overexpression immobilized GLUT4 vesicles in the TIRF zone and promoted insulin-induced GLUT4 exposure to the extracellular milieu. Myo1c also contributed to insulin-dependent actin filament remodeling. Thus we propose that interaction of vesicular Myo1c with cortical actin filaments is required for insulin-mediated tethering of GLUT4 vesicles and for efficient GLUT4 surface delivery in muscle cells.

This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

“ASCB,” “The American Society for Cell Biology,” and “Molecular Biology of the Cell” are registered trademarks of The American Society for Cell Biology.
RESULTS

Myo1c and GLUT4 associate and colocalize in submembrane vesicles and membrane ruffles

Myo1c is expressed in L6 myoblasts and myotubes and in mouse skeletal muscle (Supplemental Figure 1, A and B). Stably or transiently transfected GLUT4myc responds to insulin in myoblasts and myotubes with a greater than twofold gain at the cell surface, akin to the changes observed in mature skeletal muscle (Zaid et al., 2008). The myoblast stage affords a useful and well-characterized system to study the cell biology of GLUT4 traffic and its regulation by insulin in a muscle cell background (Ueyama et al., 1999). Insulin (100 nM, 20 min) caused enrichment in GLUT4myc at ruffled membrane areas (as reported earlier in Tong et al., 2001) and endogenous Myo1c colocalized with stably transfected GLUT4myc at these sites (Figure 1A; top panels show collapsed (compressed) focal planes, bottom panels show single focal plane). This finding is in agreement with the colocalization of the two proteins at the plasma membrane of 3T3-L1 adipocytes (Pose et al., 2004). The insulin-dependent Myo1c accumulation at sites of actin remodeling was not due to the accumulation of membrane area in them, since the ratio of endogenous Myo1c to concanavalin A (plasma membrane glycoprotein ligand) in the ruffled areas increased (Figure S1C). Myo1c was not found at perinuclear regions rich in GLUT4.

Myo1c and GLUT4myc colocalized in vesicular structures in the TIRF zone, whose dynamic behavior was examined in live myoblasts. Any puncta that were positive for GLUT4myc–green fluorescent protein (GFP) and that appeared spherical in shape, stood alone, and were clearly discernible from neighboring structures were considered “GLUT4 vesicles.” Examples of these had a median fluorescence intensity 3.1-fold above background and appeared as single diffraction-limited structures (Figure S2, A and B; n = 67 structures), detected by analysis of manually selected structures as described in Thomann et al. (2002). Such structures (termed here vesicles) were then selected for further analysis. Cotransfected GLUT4myc–red fluorescent protein fusion protein (GLUT4-RFP) and Myo1c-yellow fluorescent protein chimera (Myo1c-YFP) colocalized in both bidirectionally moving (Figure 1B, arrowheads) and stationary vesicular structures (Figure 1B, asterisks, and Supplemental Movie S1, a–c). The stationary vesicles exhibited no apparent change in their lateral position or intensity for at least 5 s, whereas mobile vesicles exhibited lateral displacements or changed their intensity due to vertical displacement in the TIRF zone (Movie S1, a–c). Within the mobile population, two types of lateral motion were detected in the time frame imaged: short-range vibrations (ranging between 0.1–3.0 μm) and short- and long-range, linear displacements of up to 10 μm.

To examine whether colocalization might have arisen as a result of Myo1c overexpression, we looked at the relative distribution of endogenous Myo1c and transiently transfected GLUT4myc-GFP (Figure 1C). Analysis of at least 15 cells per condition again revealed a colocalization between vesicular GLUT4 and endogenous Myo1c in the TIRF zone (r_{basal} = 0.64 ± 0.06, p < 0.05), where \( r \) is the Pearson’s coefficient. Insulin did not significantly change the degree of colocalization (r_{insulin} = 0.7 ± 0.03, p < 0.05).

A connection between GLUT4 and Myo1c was further made evident by the ability of the two proteins to communoprecipitate. Anti-myrm antibody-bound Sepharose beads were used to immunoprecipitate GLUT4myc from lysates of L6 myoblasts stably expressing GLUT4myc (L6-GLUT4myc cells) so as not to interfere with cytosolic regions of the transporter. Endogenous Myo1c coprecipitated with GLUT4myc but was absent in control immunoprecipitates from lysates of untransfected wild-type L6 (WT L6) cells (Figure 1D). Acute prestimulation of cells with insulin did not change the extent of

2012), the molecular machinery involved in GLUT4 vesicle tethering is unknown (Stockli et al., 2012).

Efficient GLUT4 translocation is the result of exquisite coordination of signal transduction with elaborate membrane plasticity, along with the participation of actin dynamics (Zaid et al., 2008; Chiu et al., 2011a; Foley et al., 2011; Stockli et al., 2012). Indeed, in muscle and fat cells in culture and in mature skeletal muscle and primary adipocytes, insulin induces robust actin remodeling, supporting membrane ruffles at the cell periphery (Khayat et al., 2000; Kanzaki and Pessin, 2001; Tong et al., 2001; Brozinick et al., 2004; JeBailey et al., 2004, 2007). Preventing actin remodeling with latrunculin B or cytochalasin D (Tsakiridis et al., 1994; Lopez et al., 2009) or stabilizing preexisting actin filaments with jasplakinolide (Kanzaki and Pessin, 2001; Tong et al., 2001) each precludes GLUT4 translocation, yet there is no consensus on the steps that involve actin dynamics. In adipocytes, interfering with actin polymerization impairs the final exocytic fusion step (Lopez et al., 2009), whereas in muscle cells it prevents the availability of GLUT4 near the cell periphery (Randhawa et al., 2008). In either cell type, the precise molecular events governed by actin filaments are unknown.

Actin filaments might serve as tracks for processive movement of GLUT4 vesicles, or alternatively, they may gather vesicles in an “active zone” beneath the plasma membrane for subsequent fusion. Both functions may depend on actin-associated molecular motors of the myosin family. Indeed, on one hand, members of the myosin V family that advance along actin as processive motors have been implicated in the translocation of GLUT4 to the plasma membrane in muscle and fat cells (Yoshizaki et al., 2007; Ishikura and Klip, 2008). On the other hand, the nonprocessive, class 1 myosin Myo1c (Louberry and Coudrier, 2008) was recently found to be necessary for insulin-dependent glucose uptake in skeletal muscle (Toyoda et al., 2011), and its down-regulation reduces insulin-dependent GLUT4 translocation in 3T3-L1 adipocytes (Bose et al., 2002, 2004). Class 1 myosins are particularly interesting, since they can simultaneously associate with membranes and actin filaments (Adams and Pollard, 1989; Tang et al., 2002; McKenna and Ostap, 2009), thereby regulating membrane–cytoskeleton adhesion (Nambiar et al., 2009). Accordingly, they participate in endocytosis, exocytosis, and regulation of tension between the cytoskeleton and the plasma membrane (McConnell and Tyska, 2010). Myo1c induces actin filament remodeling via its association with Rictor, independently of insulin input (Hagan et al., 2008), and it has also been proposed that Ca^{2+}- and calmodulin-dependent kinase II (CaMKII) directly phosphorylates and activates the ATPase activity of Myo1c in insulin-stimulated adipocytes (Yip et al., 2008). However, the molecular mechanism whereby Myo1c regulates GLUT4 remains poorly understood, and it is unknown whether it participates at all in GLUT4 traffic in muscle cells.

In this paper, we report that Myo1c in muscle cells associates with dynamic GLUT4 vesicles that move along linear paths in the total internal reflection fluorescence (TIRF) zone beneath the membrane and reduces GLUT4 vesicle velocity. Insulin causes immobilization of GLUT4-positive vesicles underneath the plasma membrane, and this immobilization is dependent on the presence of Myo1c and its actin-binding activity. While insulin stimulation activated CaMKII, extensive tests did not reveal involvement of this enzyme in GLUT4 surface delivery. We conclude that binding of vesicle-bound Myo1c to actin filaments underlies its participation in GLUT4 tethering within the TIRF zone for subsequent productive docking and fusion of GLUT4 vesicles with the muscle cell membrane.
The interaction between the two proteins was preserved in the presence of a high concentration of the actin-depolymerizing agent latrunculin B, indicating that their association is not mediated by actin filaments (Figure 1D). We also assessed the colocalization of Myo1c and GLUT4 in vesicular structures in isolated flexor digitorum brevis (FDB) skeletal muscle fibers. Firstly, FDB muscle fibers were cotransfected by electroporation with plasmid DNA constructs for WT-Myo1c-YFP and GLUT4-RFP and analyzed 10 d posttransfection for colocalization using two-color TIRF microscopy (Figure 1E). Analysis of at least 15 different FDB fibers from three different animals revealed that WT-Myo1c-YFP colocalized with GLUT4-RFP in many vesicular puncta (Figure 1E). In addition, FDB muscle fibers transfected with GLUT4-RFP alone were processed by indirect immunofluorescence using epifluorescence microscopy to detect endogenous Myo1c. Analysis of at least 15 muscle fibers showed colocalization of endogenous Myo1c with GLUT4-RFP-positive vesicles (unpublished data). Together the results in Figure 1 indicate that Myo1c binds to and colocalizes with GLUT4 on mobile and immobile vesicles and that

**FIGURE 1:** Myo1c and GLUT4 colocalize at the plasma membrane and on mobile intracellular vesicles. (A) Myo1c colocalizes with surface GLUT4 in insulin-induced ruffles. L6-GLUT4myc myoblasts were treated with insulin (100 nM, 10 min). Surface-localized GLUT4myc was labeled with anti-myc prior to permeabilization and Myo1c was then labeled after permeabilization. Shown are representative confocal images (of three experiments) acquired from a single focal plane 4-6 μm above the coverslip (to capture insulin-induced ruffles that may protrude 2-3 μm above the dorsal focal plane of the cell) and a combined image (compressed) of all focal planes. Arrowheads indicate Myo1c and GLUT4 sites of colocalization. In the merged image, blue artificial color represents GLUT4 staining. Scale bar: 10 μm. (B) Myo1c localizes to dynamic vesicles and colocalizes with GLUT4-positive vesicles at the TIRF zone. WT L6 myoblasts were transiently cotransfected with GLUT4-RFP (shown in red) and Myo1c-YFP (shown in green), incubated in serum-free media for 3 h, and imaged live using multicolor TIRF microscopy (Movie S1, a–c). Panel (a) shows a single frame of a representative cell in the basal state. The outlined region in panel (a) is magnified in panels (b–g), which are individual frames of this region captured 2-s apart. One stationary vesicle in which the two proteins colocalize is marked by an asterisk. Instances of colocalization on mobile vesicles are indicated by arrowheads. Scale bars: 5 and 0.8 μm (insets). (C) Endogenous Myo1c (red) colocalizes with GLUT4-GFP (green) vesicles. WT L6 myoblasts were transfected with GLUT4-GFP and treated without/with insulin as in (A); this was followed by immunofluorescence labeling of endogenous Myo1c and then multicolor TIRF microscopy imaging of fixed cells. The extent of colocalization was quantified using the Pearson’s r (R) as described in Materials and Methods. Scale bar: 5 μm. (D) Endogenous Myo1c coimmunoprecipitates with GLUT4. Lysates of serum-starved L6-GLUT4myc or WT L6 myoblasts were pretreated with/without 1 μM latrunculin B (LatB) for 1 h; this was followed by insulin stimulation as in (A). GLUT4 immunoprecipitates (IP) were immunoblotted (IB) with Myo1c antibody or anti-Myc antibody (GLUT4 myc). Shown is one representative blot out of three independent experiments. (E) Unstimulated FDB fibers expressing GLUT4-RFP and WT-Myo1c-YFP were fixed but not permeabilized. A representative TIRF image from three independent experiments is illustrated in differential image contrast (DIC), red and green channels, and as a merged image. Arrowheads mark the colocalization of the two proteins on punctate vesicular structures in the submembrane region of the muscle fiber. Scale bar: 100 μm.
reduce the recycling of fluorescently labeled transferrin (Figure S3) in 80% knockdown (not illustrated) and reduced GLUT4 trafficking by 75% (Figure 2C). Of note, down-regulation of Myo1c did not affect Akt phosphorylation on Ser473, however AS160 expression was mildly reduced, with a consequent decrease in Thr642-AS160 phosphorylation (Figure 2D), thereby maintaining the ratio of phospho-AS160 to total AS160 (Figure 2E). The decrease in AS160 expression is likely inconsequential to GLUT4 traffic, because strong siRNA-mediated knockdown of this protein increases the basal levels of surface GLUT4 (Ishikura et al., 2008; Brewer et al., 2011), yet the mild reduction in AS160 caused by siMyo1c did not alter the basal levels of surface GLUT4. In contrast, as shown above, siMyo1c knockdown virtually abolished GLUT4 translocation. These results indicate that the effect of Myo1c ablation on the insulin-dependent gain in surface GLUT4 is independent of effects on insulin signaling at the levels of Akt and AS160.

**Myo1c is not required for cytosolic dispersion of perinuclear GLUT4**

Given that Myo1c is required for the insulin-dependent gain in surface GLUT4, we asked whether it participates in the insulin-dependent movement of GLUT4 from the perinuclear region to the cell periphery (i.e., GLUT4 cytosolic dispersion) or whether it affects GLUT4 vesicles at the vicinity of the plasma membrane. GLUT4 accumulates in tubulo-vesicular structures at a perinuclear pole only partly overlapping with the transferrin receptor, giantin, and furin (respective markers of the recycling vesicles, Golgi complex, and trans-Golgi network), and insulin causes dispersion of perinuclear GLUT4 toward the cell periphery (Dugani et al., 2008). We silenced expression of Myo1c and examined the insulin-dependent perinuclear-to-cytosolic dispersion of GLUT4 GTPase transiently transfected in WT L6 myoblasts. GLUT4 GTPase distribution was analyzed by counting the number of cells in which the vast majority of GLUT4 GTPase concentrated at the perinuclear region (Figure 3A, left panel) and the number of cells with the majority of GLUT4 dispersed in the cytosol (Figure 3A, right panel). Only cells with low expression of GLUT4 GTPase were selected for analysis. As expected, insulin stimulation caused a significant increase in the percentage of cells with cytosolic GLUT4 GTPase in control siNR cells (basal 19 ± 9% vs. insulin 62 ± 5%, p < 0.05; Figure 3B). This behavior was not altered in Myo1c knockdown cells (basal 15 ± 4%, insulin 52% ± 4%, p < 0.05; Figure 3B). In contrast, down-regulation of a different motor protein also implicated in insulin-stimulated GLUT4 translocation, MyoIIA (Chung Le et al., 2009; and our unpublished data), caused a significant decrease in the percentage of cells with cytosolic GLUT4 GTPase in control siNR cells (basal 19 ± 9% vs. insulin 62 ± 5%, p < 0.05; Figure 3B). These results suggest that unlike MyoIIA, Myo1c is not required for the insulin-stimulated redistribution of perinuclear GLUT4 to the cytosol.

**Myo1c is required for GLUT4 capture at the TIRF zone**

Because loss of Myo1c did not affect cytosolic dispersion of GLUT4 vesicles but prevented exposure of GLUT4 to the extracellular medium, we inquired whether instead Myo1c affects GLUT4 vesicle accumulation beneath the plasma membrane. L6 myoblasts were cotransfected with siNR or siMyo1c along with GLUT4 GTPase, fixed, permeabilized, and labeled with anti-Myo1c antibody.

---

**FIGURE 2:** Insulin-stimulated GLUT4 translocation to the plasma membrane is reduced by Myo1c knockdown. (A) Efficient siRNA-mediated knockdown of Myo1c (200 nM, oligo 1) as indicated by immunoblotting. siNR was used as a negative control, and expression of ERK was not affected. Shown is one representative blot out of three independent experiments. L6-GLUT4 GTPase myoblasts were transfected with siNR or Myo1c siRNA (siMyo1c) and treated with/without insulin as in Figure 1. (B and C) WT L6 myoblasts transfected with siNR or siMyo1c oligo 1 (B, n = 4) or oligo 2 (C, n = 2), were transiently transfected with GLUT4 GTPase-GFP. Forty-eight hours after transfection, the cells were treated with/without insulin; this was followed by staining of surface GLUT4 GTPase and endogenous Myo1c as in Figure 1B. Knockdown cells with low expression of Myo1c were further analyzed for surface GLUT4 fluorescence intensity (at least 20 cells per condition). Quantification of fold increases in surface GLUT4 GTPase relative to the siNR basal is presented (mean ± SE, #, p < 0.05, *, p = 0.08). (D) L6-GLUT4 GTPase myoblasts stably expressing AS160 were transfected with siNR or siMyo1c and treated with/without insulin as above. Shown is one representative immunoblot for native and phospho-Akt and phospho-AS160 out of three independent experiments. (E) The pAS160/AS160 ratio from (D) is calculated from three independent experiments.

both proteins accumulate together at membrane ruffles in response to insulin.

**Myo1c knockdown reduces insulin-stimulated gain in surface GLUT4**

To determine the functional relevance of Myo1c to insulin-induced GLUT4 trafficking in muscle cells, we used targeted small interfering RNA (siRNA)-mediated Myo1c gene silencing. Treatment of myoblasts with siMyo1c achieved 75% reduction in surface GLUT4 +/−GFP. Forty-eight hours after transfection, the cells were treated with/without insulin; this was followed by staining of surface GLUT4 +/−GFP and endogenous Myo1c as in Figure 1B. Knockdown cells with low expression of Myo1c were further analyzed for surface GLUT4 fluorescence intensity (at least 20 cells per condition). Quantification of fold increases in surface GLUT4 GTPase relative to the siNR basal is presented (mean ± SE, #, p < 0.05, *, p = 0.08). (D) L6-GLUT4 GTPase myoblasts stably expressing AS160 were transfected with siNR or siMyo1c and treated with/without insulin as above. Shown is one representative immunoblot for native and phospho-Akt and phospho-AS160 out of three independent experiments. (E) The pAS160/AS160 ratio from (D) is calculated from three independent experiments.
Images of fixed, GLUT4myc-GFP–positive cells expressing normal (control) or low Myo1c levels (knockdown) were acquired using multicolor TIRF microscopy. Myo1c knockdown caused a small but significant 22.1 ± 1.5% drop (p < 0.005) in the number of GLUT4-positive vesicles at the TIRF zone (n = 25 for each condition). This suggests that Myo1c is required to capture GLUT4 vesicles at the vicinity of the plasma membrane in unstimulated cells. The effect of insulin on GLUT4 vesicle numbers in the TIRF zone was then analyzed in live cells. WT L6 cells cotransfected with GLUT4-RFP and fluorescently tagged siNR (control) or siMyo1c were imaged live before and after insulin stimulation. In siNR-transfected cells, insulin caused a 54.0 ± 3.5% decrease in the number of GLUT4-RFP vesicles in the TIRF zone relative to the basal condition (n = 6, 6–8 regions of interest [ROI] per cell; Figure S2B). This reduction is likely due to consumption of GLUT4 vesicles upon fusion with the plasma membrane, given the insulin-induced increase in surface-exposed GLUT4myc. The insulin-dependent loss of GLUT4 vesicles from the TIRF zone was greatly attenuated in cells depleted of Myo1c, which instead exhibited only a 29 ± 9% drop in GLU4 vesicle number in response to insulin relative to the basal condition (n = 4, 6–8 ROI/cell; Figure S2B). Because the number of vesicles was already reduced by Myo1c depletion prior to insulin stimulation, this finding suggests that proper capture of GLUT4 vesicles in the vicinity of the plasma membrane is a necessary pre-ambले for efficient insulin action.

A previous study reported that Myo1c regulates compensatory endocytosis following regulated exocytosis of cortical granules in Xenopus eggs (Sokac et al., 2006). To examine whether the insulin-stimulated increase in surface GLUT4 may result from a contribution of Myo1c to GLUT4 endocytosis, we quantified the effect of Myo1c knockdown on GLUT4 internalization. The results revealed that Myo1c depletion did not alter the rate of GLUT4myc internalization, which was, however, reduced by silencing expression of clathrin heavy chain, used as control (Figure 4A). In muscle cells, GLUT4 internalizes via clathrin- and clathrin-independent pathways, both requiring dynamin-2 (Antonescu et al., 2008). To further explore whether GLUT4myc vesicles colocalizing with Myo1c are exocytic or endocytic, we examined the colocalization of vesicular GLUT4myc with endogenous Myo1c upon inhibition of GLUT4 internalization by the inactive mutant of dynamin-2, Dyn2-K44A, which cannot hydrolyze GTP (Cao et al., 2000). Expression of this mutant, which blocks GLUT4 internalization (Antonescu et al., 2008) did not prevent the colocalization of Myo1c with GLUT4myc-GFP in the TIRF zone (Figure 4B). While this mutant reduced the number of GLUT4myc-positive vesicles in the TIRF zone, those remaining were still Myo1c-positive in either basal (Figure 4B, left panel) or insulin-stimulated (Figure 4B, right panel) conditions. Together, these data support the scenario that Myo1c impacts predominantly on the exocytic arm of GLUT4 traffic to the cell membrane. Consistent with this scenario, VAMP2, a component of exocytic GLUT4 vesicles (Ramm et al., 2000; Randhawa et al., 2004; Williams and Pessin, 2008; Bogan and Kandror, 2010; Xu et al., 2011), also colocalized with vesicular endogenous Myo1c (Figure S4). Finally, and further supporting the presence of Myo1c in exocytic GLUT4myc vesicles, we observed Myo1c on GLUT4myc puncta that subsequently underwent fusion with the cell membrane (Figure 4C and Supplemental Movie S2, a–c). Collectively these observations suggest that Myo1c associates with exocytic GLUT4 vesicles in the TIRF zone and is not required for GLUT4 internalization.

We next investigated whether Myo1c is required for the dynamic behavior of GLUT4 vesicles in response to insulin. WT L6 cells cotransfected with GLUT4-RFP and fluorescently tagged siNR or Myo1c siRNA. GLUT4 vesicle velocity was analyzed by TIRF microscopy of live cells before and after insulin stimulation. Three populations of GLUT4 vesicles were identified: 1) vesicles moving at < 0.1 μm/s, which showed no motion within the resolution of our detection (hereafter referred to as immobile); 2) vesicles moving in the range of 0.1–0.5 μm/s (mobile); and 3) vesicles moving at >0.5 μm/s (highly mobile). In control, unstimulated cells, 9% of GLUT4-RFP vesicles were immobile, 66% vesicles were mobile, and 25% were highly mobile (Table 1). Insulin stimulation decreased the percentage of mobile vesicles (from 66 to 46%) and increased the percentage of immobile vesicles (from 9 to 31%). Notably, in Myo1c-depleted cells, insulin stimulation increased the proportion of highly mobile GLUT4 vesicles (from 21% in control siNR-treated cells to 40% in Myo1c-depleted cells) and decreased the percentage of immobile GLUT4 vesicles (from 31 to 7% Table 1). As a result, in insulin-stimulated conditions, the average velocity of GLUT4 vesicles was higher in Myo1c-depleted cells (0.48 ± 0.03 μm/s) compared with control cells (0.36 ± 0.03 μm/s, p < 0.01). These results indicate that insulin reduces the overall mobility of GLUT4 vesicles, and Myo1c is required for the immobilizing effect of the hormone.

FIGURE 3: Myo1c is not required for cytosolic dispersion of GLUT4 to the cell periphery. (A) Representative images of the typical perinuclear localization of GLUT4myc-GFP in basal (left) and cytosolic localization in the insulin-stimulated state (right). (B and C) WT L6 myoblasts transfected with siNR, siMyo1c (B), or siMyoIIA (C), were transiently transfected with GLUT4myc-GFP. After 24 h, cells were transferred to glass coverslips and allowed to recover for 24–48 h. Cells were then treated with/without insulin for 20 min; this was followed by staining of endogenous Myo1c (B, n = 4) or MyoIIA (C, n = 3). Knockdown cells (at least 20 cells per condition) with low expression of Myo1c (B) or MyoIIA (C) were selected for further analysis of the percent of cells with cytosolic dispersion of GLUT4myc-GFP (mean ± SD, *, p < 0.01) as described in Materials and Methods.
Myo1c binding to actin immobilizes submembrane GLUT4 and underlies the contribution of the motor protein to GLUT4 translocation

Because Myo1c associated with mobile GLUT4 vesicles near the plasma membrane (Figure 1) and insulin reduced their mobility, we considered the possibility that GLUT4 anchoring into the TIRF zone occurs via the motor protein binding to the submembrane actin cytoskeleton. Accordingly, we explored whether the ability of Myo1c to simultaneously bind actin filaments and membranes is required for insulin-dependent GLUT4 traffic. To this end, we transfected L6-GLUT4/myc cells with WT-Myo1c-YFP or a truncated mutant, T-Myo1c-YFP, that can bind to membranes via its tail domain but lacks the actin-binding region and is assumed to replace the endogenous Myo1c (as used in Reizes et al. [1994]). On its own, overexpression of WT-Myo1c partially elevated surface-exposed GLUT4/myc (by 1.7 ± 0.17-fold, p < 0.05) and caused a further increase in the insulin-stimulated gain (to 5.6 ± 1.1-fold above basal from 3.6 ± 0.8-fold in GFP-expressing control cells; Figure 5A). Conversely, expression of the actin-binding–deficient Myo1c-T mutant slightly but significantly lowered basal levels of surface GLUT4/myc (to 0.7 ± 0.03) and reduced the insulin-dependent net gain (to 2.4 ± 0.6-fold compared with 3.6 ± 0.8-fold in control cells). These results are consistent with previously reported findings in 3T3-L1 adipocytes (Bose et al., 2004) and suggest that Myo1c binding to actin is required for its effect on GLUT4 translocation in both cell types. Given these results, it was necessary to address whether binding of Myo1c to actin is required for vesicular GLUT4/Myo1c colocalization and vesicle dynamic behavior.

WT L6 myoblasts were cotransfected with T-Myo1c-YFP actin binding–deficient mutant and GLUT4-RFP, and their dynamic colocalization was assessed in live cells using TIRF microscopy (Movie S2, a–c). In unstimulated cells, GLUT4-RFP and T-Myo1c-YFP colocalized on both mobile and stationary vesicles (Movie S3, a–c). As in the case of the WT-Myo1c/GLUT4 structures (Figure 1C and Movie S1, a–c), the T-Myo1c/GLUT4–positive vesicular structures displayed short-range movements and long-distance displacements (Movie S2, a–c). Hence the presence of Myo1c on GLUT4 vesicles is independent of the ability of the motor protein to bind to actin. This allowed us to investigate whether the insulin-dependent immobilization of GLUT4 vesicles requires Myo1c binding to actin filaments. L6 myoblasts were cotransfected with GLUT4-RFP and WT-Myo1c-YFP, T-Myo1c-YFP or GFP (control). As discussed above, insulin caused a mild but significant drop in the average velocity of GLUT4-RFP

FIGURE 4: Myo1c is not required for GLUT4 internalization and associates with GLUT4 exocytic vesicles. (A) The fraction of GLUT4/myc remaining at the surface at two times of internalization in L6 myoblasts transfected with siNR, siMyo1c, or siRNA to clathrin heavy chain (CHC). Main panel: insulin-stimulated cells; inset: unstimulated cells. Data are the means ± SE representative of three independent experiments. (B) Endogenous Myo1c (red) colocalizes with GLUT4/myc-GFP (green) vesicles following inhibition of GLUT4/myc internalization. L6 myoblasts were transfected with either a 5:1 M ratio of Dyn2 K44A to GLUT4/myc-GFP or GLUT4/myc-GFP alone and treated without/with insulin (100 nM, 20 min) at 24 h after transfection; this was followed by immunofluorescence labeling of endogenous Myo1c and imaging by multicolor TIRF microscopy. Arrowheads indicate examples of colocalization. Scale bar: 3 μm. (C) A GLUT4-RFP fusion event is illustrated. WT L6 myoblasts were transiently cotransfected with GLUT4-RFP (red) and Myo1c-YFP (green) and imaged live by multicolor TIRF microscopy (see also Movie S2, a–c). Individual frames shown were captured 2-ms apart. A vesicle containing GLUT4-RFP and Myo1c-YFP spreads laterally as it collapses onto the plasma membrane. Scale bars: 2.6 μm.
vesicular structures in control cells (0.33 μm/s ± 0.05 basal vs. 0.20 μm/s ± 0.04 insulin, p < 0.05; Figure 5B). Interestingly, in non-stimulated cells, overexpression of WT-Myo1c-YFP reduced the average velocity of GLUT4 vesicles (from 0.10 ± 0.01 μm/s to 0.33 ± 0.05 μm/s, respectively, p < 0.05), and this velocity was further decreased by insulin (to 0.07 ± 0.01 μm/s, p = 0.05; Figure 5B). Conversely, the average velocity of GLUT4 vesicles in unstimulated cells expressing T-Myo1c-YFP was significantly higher compared with that in WT-Myo1c-YFP-expressing cells (0.27 ± 0.03 μm/s vs. 0.10 ± 0.01 μm/s, respectively, p < 0.05). Most importantly, the average velocity of GLUT4 vesicles in cells overexpressing T-Myo1c-YFP increased significantly upon insulin stimulation (from 0.27 ± 0.03 μm/s to 0.40 ± 0.05 μm/s, p < 0.01; Figure 5B). These results indicate that insulin reduces the average mobility of GLUT4 vesicles, and Myo1c binding to actin mediates this immobilizing effect. GLUT4 vesicles binding to actin may therefore be the tethering step preceding their docking/fusion with the plasma membrane.

Myo1c knockdown prevents proper actin filament dynamics

The above results suggest that Myo1c links GLUT4 vesicle to actin filaments, and the insulin-dependent reduction in GLUT4 vesicle mobility in the TIRF zone depends on such binding. In addition, Myo1c overexpression on its own can induce cortical actin remodeling in adipocytes (Bose et al., 2004), but it is not known whether the endogenous levels of the motor protein are required for actin dynamics. Insulin causes marked actin polymerization at the cortex of L6 myoblasts (Khayat et al., 2000; Patel et al., 2003; Figure 6A, far left panel), and endogenous Myo1c colocalized with F-actin at sites of membrane ruffles (Figure 6A, marked by arrowheads). In unstimulated cells, Myo1c knockdown significantly reduced the fluorescence intensity of phalloidin-labeled stress fibers (Figure 6B, a, b, and e). Consistent with this finding, these cells displayed a slight reduction (10 ± 0.8%, p < 0.005) in cell spreading compared with control cells. Nonetheless, upon insulin stimulation (Figure 6B, c and d), cells with low Myo1c expression did not lose their ability to form actin-rich ruffles, although they experienced a significant loss of cortical actin density in such ruffles (Figure 6B) without a significant effect on actin expression (Figure 5S). By comparison, there was no change in the organization, integrity, or intensity of microtubules upon Myo1c knockdown (Figure S6). These results support the concept that endogenous Myo1c contributes to actin polymerization independently of insulin stimulation.

Because Myo1c binding to actin is necessary for GLUT4 vesicle immobilization and GLUT4 translocation (Figure 5), we hypothesized that Myo1c facilitates GLUT4 translocation in part by controlling actin polymerization at the membrane cortex. Dynamic actin polymerization depends on the availability of free high-affinity actin filament free barbed ends (FBE). L6 myoblasts transfected with siMyo1c or control siNR were stimulated with insulin and immediately permeabilized just enough to introduce rhodamine-conjugated actin monomers (Koivusalo et al., 2010). The labeled actin readily incorporated into areas of active actin remodeling in both control (87 ± 2% of cells) and Myo1c-knockdown (84 ± 2% of cells) cells (Figure 6C). Control, insulin-stimulated cells formed short and wide FBE-containing ruffles (Figure 6C) that colocalized with phalloidin-decorated F-actin (Figure 6C). The majority of insulin-stimulated cells depleted of Myo1c exhibited lamellipodia-like, contiguous, and thin FBE (42 ± 20% in siMyo1c cells vs. 9.7 ± 4% in siNR cells, p < 0.05). Nonetheless, Myo1c knockdown did not inhibit the extent of incorporation
results suggest that Myo1c does not participate in the insulin-induced generation of FBE (although it may be required for the proper arrangement of the ensuing remodeled actin filaments beneath the plasma membrane).

A connection between Myo1c, CaMKII, and GLUT4 translocation?

A recent study proposed that an insulin-dependent stimulation of CaMKII enhances the ATPase activity of Myo1c in 3T3-L1 adipocytes (Yip et al., 2008). This result was intriguing, since CaMKII is not in the canonical pathway of insulin signals, and its connection to Myo1c was of interest. CaMKII contributes to the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes (Yip et al., 2008) but notably not in skeletal muscle (Yip et al., 2008; Witczak et al., 2010). Surprisingly, the effect of CaMKII on GLUT4 traffic in either muscle or fat cells is unknown. Given the above-described requirement of Myo1c for GLUT4 translocation in muscle cells, and the participation of CaMKII in vesicle fusion events (Kolarow et al., 2007; Ciccone et al., 2008), we explored whether CaMKII is involved in GLUT4 traffic.

As a first step, we examined whether insulin leads to activation of CaMKII in muscle cells. Indeed, stimulation by the hormone led to CaMKII autophosphorylation at T286/7 (Figure S7A), which also induces partially Ca²⁺-independent “autonomous” CaMKII activity (Coultrap et al., 2010). The contribution of CaMKII to GLUT4 translocation was then assessed by a comprehensive use of pharmacological and molecular inhibitors of CaMKII, dominant-negative mutants of the enzyme, and CaMKII silencing. However, levels of surface GLUT4 were not affected by either: 1) KN-62 (Figure S8A, left panel), which prevents CaMKII stimulation by calmodulin (Tokumitsu et al., 1990); 2) the cell-penetrating inhibitory peptide tat-AC3-I (Figure S8B), which prevents CaMKII stimulation by calmodulin (Tokumitsu et al., 1990); 3) transfection of GFP-fused KIIN (an inhibitory peptide of CaMKII; Figure S8B); 4) the autacamide-3-derived peptide inhibitor AC3-I (Figure S8B), which inhibits all CaMKII activity (Coultrap et al., 2010); or 5) the combination of KIIN and AC3-I (Figure S8B). Of note, KN-62, but not CN21, reduced its both stimulated and autonomous CaMKII activity. Moreover, neither KN-62 nor CN21 affected the cell-penetrating inhibitory peptide tat-AC3-I (Figure S8B), which inhibits all CaMKII activity (Coultrap et al., 2010). The contribution of CaMKII to GLUT4 translocation was then assessed by a comprehensive use of pharmacological and molecular inhibitors of CaMKII, dominant-negative mutants of the enzyme, and CaMKII silencing. However, levels of surface GLUT4 were not affected by either: 1) KN-62 (Figure S8A, left panel), which prevents CaMKII stimulation by calmodulin (Tokumitsu et al., 1990); 2) the cell-penetrating inhibitory peptide tat-AC3-I (Figure S8B), which prevents CaMKII stimulation by calmodulin (Tokumitsu et al., 1990); 3) transfection of GFP-fused KIIN (an inhibitory peptide of CaMKII; Figure S8B); 4) the autacamide-3-derived peptide inhibitor AC3-I (Figure S8B), which inhibits all CaMKII activity (Coultrap et al., 2010); or 5) the combination of KIIN and AC3-I (Figure S8B). Of note, KN-62, but not CN21, reduced both basal and insulin-stimulated glucose uptake (Figure S7B), perhaps as a result of interactions with the glucose-binding site on GLUT4 itself. Thus, CaMKII is involved in GLUT4 traffic.

As a complement to these experiments using small-molecule or peptide inhibitors, L6-GLUT4myc cells were transiently transfected with wild-type or mutant versions of CaMKIIβ. Neither WT-GFP-CaMKIIβ, its ATP binding–impaired K43R mutant,
or a constitutively active T287D mutant affected surface GLUT4myc relative to GFP-expressing control cells (Figure S8C). However, it remained possible that other isoforms of the enzyme might be involved, since L6 myoblasts express the CaMKII α, β, γ, and δ isoforms (Figure S9). Therefore we resorted to silencing the expression of the most abundant one, CaMKIIβ. Using siRNA oligonucleotides to this isoform, we achieved a 76 ± 5% loss in total CaMKII (Figure S8D) and a significant reduction in the phospho-CaMKII signal detected by immunofluorescence. However, there was no concomitant drop in insulin-induced GLUT4 translocation under these conditions (Figure S8E).

Thus, while insulin activated CaMKII, an exhaustive test of strategies did not find any requirement for this activity in GLUT4 translocation in response to insulin. Therefore it is unlikely that CaMKII is involved in the Myo1c-dependent mobilization of the transporter to the surface of muscle cells.

**DISCUSSION**

Myo1c is a membrane-bound, actin-based motor protein that mediates membrane–cytoskeletal interactions and has been implicated in diverse phenomena involving vesicle traffic including exocytosis, endocytosis, cell migration, and in the generation of plasma membrane tension (Barylko et al., 2005; McConnell and Tyska, 2010). Recent studies show that silencing Myo1c expression partly reduces insulin-mediated glucose uptake into skeletal muscle (Witzczak et al., 2010) and lowers GLUT4 translocation in 3T3-L1 adipocytes (Bose et al., 2002, 2004; Yip et al., 2008). In this study, we provide evidence for the molecular mechanism underpinning this response. This was shown in skeletal muscle cells, given the prominence of skeletal muscle in the physiological uptake of glucose from the blood.

We first show that Myo1c is not present in the perinuclear region, where the majority of GLUT4 is located in resting cells. However, the two proteins colocalize in peripheral areas of the cell. Within the 200-nm reach of the inner surface of the plasma membrane (the TIRF zone), Myo1c and GLUT4 colocalize on vesicular structures in both L6 myoblasts and isolated single fibers of FDB muscle. Based on their velocity, these GLUT4/Myo1c vesicles distribute into three classes. Insulin increased the proportion of immobile vesicles within the TIRF zone, and effectively reduced the GLUT4 vesicles’ average velocity. This behavior was lost upon Myo1c depletion, causing an increase in the average vesicular velocity, which concomitantly prevented exposure of GLUT4 at the outer face of the membrane. Moreover, Myo1c depletion reduced the amount of GLUT4 vesicles at the TIRF zone, but not their spreading from perinuclear to cytosolic distribution, suggesting that Myo1c-dependent immobilization retains GLUT4 vesicles beneath the membrane. Indeed, overexpressing Myo1c decreased the average mobility of GLUT4 vesicles in the TIRF zone, concomitant with potentiation of insulin action on GLUT4 translocation. These effects were lost when a mutant Myo1c unable to bind actin filaments, rather than WT Myo1c, was transfected. We propose that Myo1c helps tether GLUT4 vesicles to submembrane actin filaments, thereby reducing their mobility, as a prerequisite to GLUT4 vesicle docking and fusion with the plasma membrane to enact increased glucose uptake.

**Characteristics of GLUT4 vesicle dynamics and effect of insulin**

GLUT4 vesicles in the TIRF zone could be segregated into three groups, depending on whether they have negligible, medium, or high velocity. The proportion of these pools of vesicles was 9, 66, and 25% in resting cells, respectively. Insulin increased the proportion of immobile vesicles, changing the distribution to 31, 46, and 23%, and, consistently, decreased the overall vesicular average velocity (from 0.33 μm/s ± 0.05 to 0.20 μm/s ± 0.04). Insulin stimulation also reduced the number of GLUT4 vesicles in the TIRF zone, presumably because they are consumed by fusion with the plasma membrane faster than they arrive at this zone. These findings are consistent with change in the speed and number of GLUT4 puncta in isolated FDB muscle fibers in response to insulin (Lizunov et al., 2012).

Further, we demonstrate for the first time that Myo1c and GLUT4 move on the same puncta in the TIRF zone (Figure 1 and Movie S1, a–c). This suggests that Myo1c participates in GLUT4 traffic events in this zone. Accordingly, Myo1c depletion promoted GLUT4 vesicle mobility, elevating the proportion of highly mobile vesicles from 21% in the corresponding control cells to 40%. This resulted in an increase in the average vesicular velocity from 0.36 ± 0.03 μm/s to 0.48 ± 0.03 μm/s. We speculate that such a rise in vesicle mobility may result from loss of binding of vesicular Myo1c to actin filaments.

Notably, the range in GLUT4 vesicle speeds in myoblasts was similar to that of mobile GLUT4 vesicles in isolated FDB muscle fibers from transgenic mice with muscle-specific expression of hemagglutinin (HA)-GLUT4-GFP (Lizunov et al., 2012). In both systems, the vesicle velocity was heterogeneous and ranged from 0.1 to 2 μm/s with no apparent preferential directionality (Lizunov et al., 2012; Table 1). In contrast to our findings, however, Lizunov et al. (2012) found that most of the HA-GLUT4-GFP structures were stationary, with only 10% exhibiting displacements exceeding 1 μm (their limit of detection). That limit may have missed the vibrational movements that we detected in myoblasts (detection limit of 0.1 μm). Differences may also arise from the complexity of mature muscle fibers relative to myoblasts, as the space and vesicular movement in the former may be restricted by the abundance of contractile fibers.

It is important to note that the number of GLUT4 vesicles in the TIRF zone decreased in unstimulated cells depleted of Myo1c, possibly as a result of diminished tethering. Hence Myo1c determines GLUT4 vesicle mobility, even in the absence of insulin. This effect is exacerbated in the insulin-stimulated state, in which Myo1c depletion precludes the insulin-dependent drop in vesicle number and mobility exerted by the hormone. We speculate that, in Myo1c-containing cells, the insulin-induced polymerization and remodeling of actin filaments beneath the plasma membrane increases the probability of vesicle immobilization. This possibility is supported by the observation that a Myo1c mutant unable to bind actin filaments still bound to GLUT4 vesicles but prevented the insulin-dependent reduction in vesicle mobility and concomitantly abated GLUT4 insertion in the membrane (Figure 5).

**Effect of Myo1c on actin dynamics**

Myo1c overexpression causes actin polymerization in adipose cells by association with Rictor (Hagan et al., 2008), but the mechanism and implication of this phenomenon remains obscure. Because overexpression might induce a nonphysiological response, we tested whether, conversely, Myo1c depletion would prevent actin polymerization (documented by measuring actin monomer incorporation into filament FBE). Whereas the net amount of actin incorporation into FBE was not altered by Myo1c depletion, Myo1c-depleted cells showed thinner insulin-induced lamellipodia-like ruffles that run extensively along the cell periphery, compared with the short and dense ruffles observed in insulin-stimulated control cells. We surmise that Myo1c knockdown precludes proper localization of
FBE and that normally Myo1c directs actin monomer incorporation to specific loci beneath the plasma membrane. Myo1c knockdown also reduced the net amount of polymerized actin. The molecular mechanism by which Myo1c controls actin dynamics is not known, but it may be mediated via Arp2/3 binding, as is the case for the fungal Myo1 orthologues (Evangelista et al., 2000; Lechler et al., 2000), or Myo1c may compete with the binding of F-actin–severing proteins (e.g., cofilin), preventing proper actin dynamics, among other possibilities.

Interestingly, Myo1c knockdown also reduced the intensity of stress fibers in unstimulated cells. Because there was a decrease in the number of vesicles in the TIRF zone under these conditions, yet surface levels of GLUT4 were not altered in the absence of insulin treatment, stress fibers may contribute to providing GLUT4 vesicles to the vicinity of the plasma membrane in the basal state (as seen in Figure 1). Whether stress fiber integrity/formation would allow GLUT4 mobility toward the cell periphery requires future investigation.

**Steps in GLUT4 traffic controlled by Myo1c**

The perinuclear-to-cytosolic dispersion of GLUT4 induced by insulin was preserved in Myo1c knockdown cells. This is consistent with this step being mediated by microtubule- but not actin-based movement, in analogy with other systems (Nascimento et al., 2003). In contrast, in Myo1c knockdown cells, the net amount of GLUT4 vesicles in the TIRF zone was reduced, suggesting GLUT4 vesicles arriving at this zone cannot be retained by the normal mechanisms.

Most models of GLUT4 traffic propose that vesicles reaching the plasma membrane emanate from "storage" or "primed" pools (Blot and McGraw, 2008; Brewer et al., 2011) and suggest a tethering step from whence docking and fusion proceed. However, the role of tethering in this progression is unknown. While we propose that Myo1c tethers GLUT4 vesicles to actin filaments, we can only speculate on the consequence of this step. Tethered/immobilized vesicles may have more opportunity for efficient interaction with membrane proteins, allowing the participating vesicular VAMP2 to effectively engage with syntaxin4 and SNAP23 on the plasma membrane (SNARE proteins have been shown to mediate fusion of insulin-sensitive GLUT4 vesicles with the membrane [Foster and Klip, 2000; Williams and Pessin, 2008]). The presence of Myo1c in ruffled regions might also indicate a Myo1c association with the plasma membrane, potentially through inositol phospholipids (Hokanson et al., 2006) or plasma membrane–associated proteins (Chen et al., 2007). Whether Myo1c also regulates docking and fusion with the plasma membrane requires further investigation.

Insulin regulates the Myo1c ATPase activity in 3T3-L1 adipocytes (Bose et al., 2004) and causes Myo1c phosphorylation at a site that binds calmodulin light chains (Yip et al., 2008). This suggested that phosphorylation mediated by CaMkII would activate Myo1c and, consistent with this prediction, inhibition of the kinase reduced insulin-stimulated glucose uptake in adipocytes (Yip et al., 2008), although GLUT4 translocation was not assessed. These observations were intriguing and prompted us to probe the potential role for CaMkII in insulin-stimulated GLUT4 traffic in muscle cells. Interestingly, insulin promoted CaMkII phosphorylation at the autoregulatory site of the kinase that would relieve autoinhibition (Bayer et al., 2001, 2006; Figure S7A). CaMkII also colocalized and communoprecipitated with α-actinin-4 (Supplementary Figure S7, C and D), a protein we had previously shown to interact with GLUT4 and actin and to be required for normal GLUT4 traffic to the plasma membrane (Foster et al., 2006; Talior-Volodarsky et al., 2008). We had hypothesized that CaMkII—possibly acting through α-actinin—might be required for insulin-dependent GLUT4 translocation. However, this link did not materialize, as an exhaustive strategy to inhibit the CaMkII or deplete its expression was inconsequential to GLUT4 translocation (Figure S8), which nonetheless required Myo1c.

In conclusion, we present evidence that insulin reduces the mobility of GLUT4 vesicles within the TIRF zone, and Myo1c is required for such immobilization through its association with submembrane actin. Concomitantly, delivery of GLUT4 for docking/fusion may be facilitated by the Myo1c–enhanced polymerization of actin filaments in this region. In this manner, Myo1c contributes to insulin-dependent GLUT4 exposure at the surface of muscle cells.

**MATERIALS AND METHODS**

**Reagents, siRNA, and constructs**

Monoclonal anti-myc antibody and monoclonal anti-CaMkII were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti–phosphorylated Akt (Ser473), anti-Akt, ERK1/2 antibodies were from Cell Signaling Technology (Beverly, MA). Fluorescein isothiocyanate (FITC) conjugate of monoclonal anti-tubulin (clone DM1A) and polyclonal anti-Myo1c were from Abcam (Cambridge, MA). Polyclonal anti–phosphorylated Thr286/7-CaMkII was from Phosphosolutions (Aurora, CO). Rabbit polyclonal anti-α-actinin-4 antibody was from Alexis Biochemicals (San Diego, CA). Anti-AS160 and anti–phosphorylated Thr642 AS160 were from Millipore (Billerica, MA). Anti–γ-actin, anti–α-SMA (smooth muscle actin), and anti–β-actin antibodies were kindly provided by Boris Hinz (University of Toronto, Toronto, ON). Monoclonal anti-HA antibody (clone HA.11) was from Covance (Berkeley, CA). Alexa Fluor 488–ConA conjugate, Alexa Fluor 633–phalloidin, and Alexa Fluor 488–phalloidin were purchased from Molecular Probes (Eugene, OR). Human insulin was purchased from Eli Lilly (Indianapolis, IN). Collagenase IV was from Worthington Biochemical Corporation (Lakewod, NJ). Pheno-red free BD Matrigel Matrix was from BD Biosciences (Bedford, MA). YFP-tagged YFP-Myo1c and YFP-Myo1c (T) plasmids were kindly provided by Michael P. Czech (University of Massachusetts Medical School, Worcester, MA). Expression vectors containing GFP conjugated to the CaMkII inhibitory AC3-I (KKALHRQEAVDCL) or the CaMkII control AC3-C (KKALHAQERVDCCL) peptides were kindly provided by Mark E. Anderson (University of Iowa, Iowa City, IA). A plasmid coding for the CaMkII inhibitory peptide KIIN (KRPPKGLQGLGRAVIIEEDDRDDVLK) (Chang et al., 2001) fused to GFP (GFP-KIIN) was obtained from T. R. Soderling (Oregen Health and Sciences University, Portland, OR). Plasmids, encoding GFP-CaMkIIβ wild-type and its GFP-tagged mutants A303R (CaM-binding impaired), K43R (ATP-binding impaired), and T287D (constitutively active), as well the CaMkII inhibitor peptide tet-CN21 (tetcN21) and the scrambled sequence control (tetCtrl), were as previously described (Vest et al., 2007). Dynamin-2 K44A cDNA was a kind gift from Mark McNiven (Mayo Clinic, Rochester, NY). GLUT4-RFP was generated by amplification of GLUT4 fragment from GLUT4myc-GFP (Ueyama et al., 1999) and subcloning into pmCherry-N1 (Clontech, Mountain View, CA) using EcoRI/AgeI restriction sites. VAMP2-HA was generated by amplification of VAMP2 fragment from VAMP2-GFP (Randhawa et al., 2008) and subcloning into pcXIIH using AgeI-Pacl restriction sites. siRNAs targeted against Myo1c (5′-atactaggtggagcagacta-3′, and 5′-caggctgaatctgtgat-3′), MyoIIA (5′-aaggtagtgagctagcatt-3′), clathrin heavy chain-1 (5′-aac cgg aag tta gta tat-3′) (Antonescu et al., 2008), and non–related control (5′-ataaagctagatagagat-3′) were purchased from Qiagen (Chatsworth, CA) and named siMyo1c, siMyoIIA, and siNR. ON-TARGETplus SMARTpool siRNA targeted against CaMkIIβ were
obtained from Dharmaco (CaMKIγ; accession number L-099520; #1, 5′-gtaatcaugtctaccta-3′; #2, 5′-gtaggaatggcagaata-3′; #3, 5′-gtagctggtcgaagaat-3′; #4, 5′-cagctcauggtgaaa-3′; Lafayette, CO).

Cell culture and transfection
Rat L6 myoblasts without (WT L6) or with stable expression of myc-tagged GLUT4 L6-GLUT4myc were cultured as described previously (Ueyama et al., 1999). To create L6-GLUT4myc myoblasts stably expressing human AS160 (cDNA a kind gift from G. E. Lienhard, Dartmouth Medical School) or VAMP2-HA, we cloned the cDNAs into pQXIP or pQCXIH retroviral vectors (BD Biosciences), respectively. Retroviral supernatants were produced in Phoenix packaging cells obtained from G. P. Nolan (Stanford University, Stanford, CA) according to their protocol (www.stanford.edu/group/nolan/index.html) and used to infect L6-GLUT4myc myoblasts. Forty-eight hours postinfection, the cultures were treated with 2 μg/ml puromycin (for L6-GLUT4myc-AS160) or 200 μg/ml hygromycin (for L6-GLUT4myc-VAMP2-HA) for 7 d to create a stable pool of clones (L6-GLUT4myc-AS160) that were cultured in the same manner as L6-GLUT4myc cells. Transfection of siRNAs or cDNA plasmid constructs was performed with Lipofectamine RNAiMAX or Lipofectamine 2000, respectively, as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). Transfection of siRNA proceeded for 24 h and of cDNA for 4–6 h; cells were then allowed to recover or were trypsin-treated and transferred to glass coverslips 24–48 h before experimentation.

GLUT4myc immunoprecipitation and immunoblotting
L6-GLUT4myc myoblasts seeded in 10-cm dishes were grown to confluence and serum-deprived for 3 h before stimulation with 100 mM insulin for 20 min. Lysates (1.5 mg protein) were immunoprecipitated with 40 μl of 9E10 Monoclonal Affinity Matrix as previously described (Foster et al., 2006). Cells were washed quickly with cold phosphate-buffered saline (PBS), and lysed in 1× Laemmli sample buffer supplemented with a cocktail of protease and phosphatase inhibitors (NaF 1 mM and Na3VO4 1 mM). Samples were resolved by 10% SDS–PAGE and subjected to immunoblotting.

Glucose uptake, GLUT4 translocation, and GLUT4 internalization in cell monolayers
L6-GLUT4myc cells grown in 24-well plates and serum-starved for 3–5 h were treated as indicated in the figure legends and incubated with or without 100 nM insulin for 20 min. uptake of 2-deoxy-[14C]glucose was then measured as previously described (Tamrakar et al., 2011). Nonspecific uptake was determined in the presence of cytochalasin B (20 μM) and subtracted from all values. GLUT4myc internalization was measured as described earlier (Antonescu et al., 2008). Surface GLUT4myc levels were determined in paraformaldehyde-fixed, nonpermeabilized L6-GLUT4myc myoblast monolayers (grown in 24-well plates) labeled with anti-myc antibody, which was followed by HRP-conjugated secondary antibody, as previously described (Ueyama et al., 1999; Tamrakar et al., 2011).

Transferrin recycling assay
L6-GLUT4myc cells were serum-starved for 2 h and then incubated with 50 μg/ml of AS55-conjugated transferrin (Invitrogen) in serum-free medium supplemented with 1% bovine serum albumin (BSA), without or with insulin (100 nM) for 10 min at 37°C. One plate of cells was placed on ice; washed twice with PBS, twice with acid buffer (0.15 M NaCl, 0.1 M glycine, pH 3.0), twice with PBS; and fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature. A second plate was washed with 500 μg/ml holo-transferrin (Sigma-Aldrich, St. Louis, MO) for 10 min at 37°C with or without insulin, washed, and fixed as above. All cells were quenched with 0.1 M glycine for 10 min, washed once with PBS, and processed for fluorescence detection of AS55-conjugated transferrin in single cells.

Fluorescence microscopy and cell-surface GLUT4myc detection in single cells
Immunofluorescence detection of surface GLUT4myc in L6-GLUT4myc myoblasts was performed as previously described (Torok et al., 2004). After 3-h serum starvation, cells were stimulated without and with insulin (100 nM) for 10 min at 37°C. Cells were quickly washed twice with cold PBS, fixed with 3% (vol/vol) PFA, and blocked with 5% (vol/vol) milk. Surface GLUT4myc was stained with anti-myc primary antibody, which was followed by secondary antibody. For surface GLUT4myc detection in single cells, WT L6 cells were transiently transfected with GLUT4-RFP or GLUT4myc-GFP, fixed as above but not permeabilized, and incubated with monoclonal anti-myc antibody and Cy5-conjugated secondary antibody. GLUT4 translocation was calculated as the ratio of Cy5 to total RFP or GFP fluorescence intensity per cell, in 20 cells per condition. For detecting surface GLUT4myc and intracellular Myo1c or CaMKII, surface GLUT4myc was first labeled as above; this was followed by membrane permeabilization with 0.5% Triton X-100 for 3 min, for subsequent Myo1c or pThr286/7 CaMKII immunolabeling. For double Myo1c and F-actin or pThr286/7 CaMKII labeling, cells were fixed with 3% PFA in PBS for 10 min on ice and an additional 10 min at room temperature, then permeabilized with 0.5% Triton X-100 for 3 min and stained with Alexa Fluor 488–phalloidin, anti-Myo1c antibody, or anti-pThr286/7 CaMKII antibody, as indicated. For ConA labeling of the cell surface, cells were fixed with 3% PFA for 20 min, incubated with ConA (50 μg/ml PBS) at 4°C for 30 min, and fixed with 3% PFA for 20 min at room temperature. Cells were then permeabilized with 0.5% Triton X-100 for 3 min and stained with an anti-Myo1c antibody. For double tubulin/Myo1c staining, cells were fixed for 10 min in cold (−20°C) methanol and then labeled with FITC-conjugated anti-tubulin and anti-Myo1c antibodies. Fluorescence images were acquired on a Zeiss LSM 510 META laser-scanning confocal microscope (Thornwood, NY), using a 60×/1.4 numerical aperture (NA) Plan-Apochromat objective and Zeiss AIm software. 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 LSM5 Image software. Images were converted into TIFF files by ImageJ version 1.42 (National Institutes of Health [NIH]), http://rsb.info.nih.gov/ij software and arranged into figures using Adobe Photoshop (San Jose, CA).

Image analyses were analyzed using ImageJ to quantify fluorescence intensities from unmanipulated raw images. For Figures S1C and S6B, ROIs on the plasma membrane (ruffles), inside the cell (cytosol), or the entire cell plus an area outside the cell (background) were manually traced, and their mean fluorescence intensities were quantified after background subtraction. Vessel number at the TIRF zone in fixed cells was analyzed using the Find 2D spots function of the Velocity 5.5 package (Improvision, Lexington, MA) and applied to the polygon drawn around the cell. Before analysis, images were adjusted for better vesicle segmentation, and the vesicle number in the evanescent field was normalized to total GLUT4myc-GFP epifluorescence (vesicle number in TIRF zone/GLUT4-GFP epifluorescence ratio). For colocalization analysis, TIRF images were acquired at high magnification (150×/1.45 NA objective), and ROIs
containing vesicular GLUT4myc-GFP were cropped from the entire image. The degree of colocalization was quantified by Pearson’s coefficient of correlation (R) using the Velocity colocalization measurement tool and subjected to Student’s t test statistical analysis. The manual track function of the Velocity 6.1 package was used to analyze GLUT4 vesicle velocity and displacement at the TIRF zone. Single GLUT4 puncta were marked as follows: the particle center was manually located and traced for the indicated time intervals or until its disappearance from the TIRF zone. Data are presented as means ± SEM. Statistical significance was analyzed using Student’s t test.

For cell spreading quantification, L6 myoblasts were fixed, the actin cytoskeleton was labeled with phallloidin, and images of mock and Myo1c-depleted cells were captured by confocal fluorescence microscopy, which was followed by image deconvolution. The mean cellular area of cells was measured with Velocity 5.5.

**Video microscopy**

For studying the colocalization between GLUT4-RFP and WT-Myo1c-YFP or T-Myo1c-YFP (Movies S1 and S2 and Figure 1C) in multicolor TIRF microscopy, WT L6 cells on 18-mm #1.5 round glass coverslips (Zeiss) were transfected and analyzed 24 h after transfection. Thirty minutes before observations, the medium was switched to HEPES-containing RPMI-1640. Images were acquired on an Olympus IX81 microscope equipped with a dual-laser (488 nm, 561 nm) CellTIRF temperature control unit using the 150×/1.4 NA objective and a QuantEM back-thinned EM-CCD. The penetration of the evanescent field was measured to be 110 nm. Images were acquired using MetaMorph and CellCut acquisition software and converted into TIFF files by imageJ software (NIH). Images were filtered using the Unsharp Mask plug-in of imageJ and converted to movies.

For study of GLUT4-RFP vesicle dynamic behavior, WT L6 myoblasts were transfected with the listed siRNA oligonucleotides, replated on 18-mm #1.5 round glass coverslips (Zeiss), cultured for 24 h, and then transfected with indicated cDNA constructs (GLUT4-RFP/WT-Myo1c-YFP/T-Myo1c-YFP) for an additional 24 h. Cells were serum-starved for at least 3 h, the culture medium was switched to HEPES-containing RPMI-1640, and the cells were imaged on a Olympus IX81 microscope equipped with a dual-laser CellTIRF unit, as above, using Velocity 4.3.2 build 23 and CellCut acquisition software. Individual cells were imaged in the basal state for the indicated time intervals, the acquisition was stopped, and insulin was added for 10 min (final concentration 100 nM). Imaging of the same cells continued for indicated time intervals.

**Actin-free barbed-end assay**

Filament barbed end (FBE) were determined by a modification of previously described methods (Chan et al., 1998; Frantz et al., 2008; Koivusalo et al., 2010). Briefly, serum-starved myoblasts on coverslips were incubated with or without with insulin (100 nM) for 20 min. Cells were permeabilized for 15 s in 20 mM HEPES, 140 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM EGTA, 5 mM glucose, 1% BSA, and 0.5 mM ATP (pH 7.5) containing 0.04% saponin and 0.02 μg/μl rhodamine-labeled rabbit skeletal muscle actin. After 15 s, the solution was diluted threefold with permeabilization buffer without saponin and rhodamine-actin, and incubation continued for 3 min, followed by fixation in 3% PFA (20 min). For comparison of actin FBE formation in control and Myo1c knockdown cells, the area encompassing each single, continuous, rhodamine-labeled ruffle was manually traced on highly magnified images using the freehand tool, the ruffle mean fluorescence intensities were measured, and background subtraction using confocal fluorescence microscopy was done. The fluorescence intensity is reported as the ratio of the fluorescence of the ruffle relative to that of the cytosol. Images illustrated were acquired by epifluorescence.

**Detection of CaMKII isoforms and Myo1c expressed in L6 cells by reverse transcriptase PCR (RT-PCR)**

Excision of rat muscle was approved by the Animal Care Committee of the Hospital for Sick Children. Total RNA (200 ng) from rat skeletal muscle, L6-GLUT4myc myoblasts, and myotubes was prepared using guanidine isothiocyanate-phenol-chloroform and used for one-step RT-PCR, according to the manufacturer’s instructions (Qiagen, Valencia, CA). Primers were designed using Primer3 software (CaMKIIα: forward (FWD) 5′-ctgaaccccctccatccacct-3′ and reverse (REV), 5′-acaatgcctggatttcctctct-3′; CaMKIIβ: FWD, 5′-gaaagagccgagggctcgcag-3′ and REV, 5′-gattggtctgctggaaacctt-3′; CaMKIIγ: FWD, 5′-gcgtcagaaagagaaagtgc3′ and REV, 5′-ctgctcctcaaggaaggattgg-3′), (CaMKIIα: FWD, 5′-ctgctcctcaaggaaggattgg-3′ and REV, 5′-gtgtccctcagctttcagc-3′) and (GAPDH as a control FWD, 5′-tgccctcagccgtgctt-3′ and REV, 5′-tccagctggtggtggtc-3′) and used in the following PCR program on Thermal Cycler (Bio-Rad, Hercules, CA): RT at 50°C/30 min, denaturation at 95°C/15 min and repeated cycles of denaturation at 94°C/45 s, annealing at 57°C/45 s, and extension at 72°C/45 s, followed by final extension at 72°C/10 min. After RT, CaMKIIα, β, γ, δ and GAPDH were amplified for 35 (CaMKIIα, β, γ, δ) and 29 (GAPDH) cycles, after titration for each gene-specific primer pair to ensure linearity.

**Analysis of isolated muscle fibers from adult mice**

All animal-use protocols were approved by the Hospital for Sick Children’s Animal Care Committee in accordance with the Canadian Council of Animal Care guidelines. WT-Myo1c-YFP and/or GLUT4-RFP plasmids (total ~20 μg, at 1 μg/μl) were injected subcutaneously into the FDB muscle of male mice (12 wk old); this was followed by electroporation as previously described (DiFrancesco et al., 2009). Twenty-20 ms pulses of 100 V with 980-ms intervals were delivered using a CUY21 EDIT square electroporator (TR-Tech, Tokyo, Japan). Mice were killed 10 d later, and FDB muscles were obtained by enzymatic digestion of the whole muscle with type IV collagenase (600 U/ml, 90 min), followed by mechanical dissociation with fire-polished Pasteur pipettes. Isolated FDB fibers were plated on Matrigel-coated coverslips (diluted 1:4 in serum-free DMEM). After 2.5 h, fibers were washed gently with PBS and fixed with 4% PFA/CBS for 20 min at room temperature, washed again in PBS, and quenched with 0.125 M glycine (10 min). Fibers cotransfected with YFP-Myo1c and GLUT4-RFP were visualized under a two-color TIRF microscope, as described above. For visualization of GLUT4-RFP with endogenous Myo1c, isolated muscle fibers were fixed as above, permeabilized with Triton 0.1%/PBS, and QuantiFISH. Images were acquired by epifluorescence microscopy.

**ACKNOWLEDGMENTS**

This work was supported by grant MT12601 to A.K. from the Canadian Institutes of Health Research (CIHR). S.B. was supported by fellowship from the Canadian Diabetes Association and the Banting and Best Diabetes Center. T.C. was supported by a Banting Scholarship from CIHR. C.O.-F. was supported by visiting doctoral scholarship UCH0713 from MECESUP. We thank Zhi Liu for technical assistance in this study; ShuheI Ishikura for...


SUPPLEMENTARY MATERIAL:

**Supplementary movies 1. (a-c)** Time-lapse recording of wild-type L6 myoblasts expressing GLUT4-RFP (movie 1a) and Myo1c-YFP (movie 1b) using multicolor TIRF microscopy (merged in movie 1c). Cells were observed 24 h after transfection in non-stimulated conditions. Video represents 1.7 min of recording. Note that the two proteins colocalize in stationary and dynamic vesicles.

**Supplementary movies 2. (a-c)** Time-lapse recording of a GLUT4 vesicle fusion event in wild-type L6 myoblasts co-expressing GLUT4-RFP (movie 2a) and Myo1c-YFP (movie 2b) using multicolor TIRF microscopy (merged in movie 2c). Cells were imaged 24 h after transfection, in non-stimulated conditions. This movie shows the fusion event described in Figure 4 and is encoded for real time playback (*i.e.*, 7 fps). Note that the two proteins colocalize at the surface of GLUT4 vesicles just prior to fusion with the plasma membrane.

**Supplementary movies 3. (a-c)** Time-lapse recording of wild-type L6 myoblasts expressing GLUT4-RFP (movie 3a) and actin binding deficient mutant Myo1c-T-YFP (movie 3b) were transfected as in movie 1 and analyzed using multicolor TIRF microscope; merged (movie 3c). The video represents 1.5 min of recording. Note that GLUT4 and the Myo1c truncated proteins colocalize in stationary and dynamic vesicles.
Supplementary figure 1. Myo1c is expressed in L6 myoblasts and myotubes and accumulates in insulin induced ruffles. (A) Total RNA was isolated from L6-GLUT4myc myoblasts (Mb) or myotubes (Mt) and used for RT-PCR detection of Myo1c transcript, as described in Materials and Methods. (B) Quantities of Myo1c protein in L6-GLUT4myc myoblasts, mouse soleus (SOL), extensor digitorum longus (EDL), and tibialis anterior (TA) skeletal muscles, and fat (F) tissue. (C) Insulin induces Myo1c accumulation in membrane ruffles. Upper panel: L6-GLUT4myc myoblasts were treated with 100 nM insulin for 20 min at 37°C, fixed and then stained with ConcanavalinA (ConA) (to label the plasma membrane) and anti-Myo1c antibody. Shown are representative confocal microscopy images. White boxes outline examples of insulin-induced ruffle (**) and non-ruffle (*) regions. Numerous ruffle (arrows) and non-ruffle (arrowheads) regions were analyzed for fluorescence intensity in each cell (3-8 different regions), as indicated in this representation. Scale bar: 10 µm. Lower panel: Quantification of the insulin-induced fold increases in fluorescence intensity ratio of Myo1c signal relative to ConA in ruffle/non ruffle regions for 20 cells, analyzed by NIH ImageJ software. Data are expressed as mean ± SEM.
Supplementary figure 2. (A) Manual selection of GLUT4-RFP puncta for analysis. Wild-type L6 myoblasts expressing GLUT4-RFP were subjected to time-lapse total internal reflection fluorescence microscopy (TIRF-M). (A) Single frame of a cell in the basal (unstimulated) condition. Red circles point to the typical, manually selected puncta: These were small, bright structures that appear spherical, clearly discernible from neighboring structures, observed in a minimum of 20 sequential frames (not shown).

(B) The pixel intensities corresponding to manually selected puncta were subjected to Gaussian fitting, as described (Thomann et al., 2002). Shown is the intensity of such GLUT4 puncta, after background subtraction, relative to the amplitude of the Gaussian fit. These puncta had a median fluorescence intensity of 3.1-fold over background (n=67 puncta). Analysis of the residuals of the Gaussian fit of each structure revealed that the majority of manually selected puncta (41/67 or 61%) are single diffraction-limited structures and are thus likely to be single GLUT4 vesicles.
(C) The insulin-stimulated reduction in the number of GLUT4-RFP vesicles in the TIRF zone is attenuated by Myo1c knockdown. WT L6 cells were co-transfected with GLUT4-RFP and fluorescently labeled siNR or siMyo1c, and TIRF microscopy was used to visualize and image the GLUT4-RFP puncta of live cells before and after insulin stimulation (100 nM, 10 min). Using the criteria in A, B above, the number of vesicles in the TIRF zone before and after insulin was analyzed in the same regions of interest (ROI) of 10 µm by 10 µm, using the Find 2D spots function of the Velocity 5.5 package. Before analysis, images were adjusted for better vesicle segmentation. The figure indicates the percent reduction (insulin relative to basal) in the number of GLUT4-RFP vesicles in the TIRF zone, for siNR-control cells (54.0 ± 3.5%, n=6, 6-8 ROI/cell) or siMyo1c-transfected cells (29 ± 9%, n=4, 6-8 ROI/cell). Scale bar: 4 µm.
Supplementary figure 3. Myo1c knockdown does not alter transferrin recycling. L6-GLUT4myc cells were transfected with fluorescently labeled siNR or siMyo1c, and transferrin recycling was assessed 48 h after transfection, by single cell fluorescence detection of A555-conjugated transferrin signal as described in “Materials and Methods”. Data are expressed as the fraction of A555-conjugated transferrin signal remaining in the cells after the 10 min chase with holo-transferrin, relative to the start of the chase (n=3). Statistical analysis by two-way ANOVA showed no difference between siNR and siMyo1c in either basal or insulin-simulated conditions.

Supplementary figure 4. Endogenous Myo1c (red) colocalizes with VAMP2-HA (green) positive structures. WT L6 myoblasts stably expressing VAMP2-HA were fixed, processes for indirect immunofluorescence with an anti-Myo1c and anti-HA antibody followed by secondary antibodies conjugated with Cy3 and Alexa488, respectively and then imaged using multicolor TIRF microscopy. Scale bar: 11 μm.
Supplementary figure 5. Myo1c knockdown does not lead to change in expression of the major actin isoforms expressed in wild-type L6 myoblasts. Cells were transfected with 200 nM siNR or siMyo1c and stimulated or not by insulin (100 nM, 20 min). Cell lysates were resolved by SDS-PAGE and immunoblotted with indicated antibodies to γ-actin, α-smooth muscle actin (α-SMA), or β-actin. Shown are representative blots from three independent experiments.
Supplementary figure 6. Down-regulation of Myo1c does not affect microtubule’s organization and integrity. L6-GLUT4myc myoblasts were transfected with siNR or siMyo1c, treated with or without insulin (100 nM, 10 min), labeled with antibodies to Myo1c and β–tubulin and analyzed by confocal fluorescence microscopy. Representative images of three independent experiments are shown. Arrowheads mark some cells with low expression of Myo1c. Scale bar: 10 µm.
Supplementary figure 7. (A) Insulin causes CaMKII phosphorylation in response to insulin stimulation in L6 myoblasts. L6-GLUT4myc myoblasts were serum-starved for 3 h before insulin stimulation (100 nM) for the indicated time periods. Total lysates were immunoblotted with indicated antibodies and representative blots from three independent experiments are shown. (B) CaMKII inhibitors reduce insulin-stimulated glucose uptake. L6-GLUT4myc myoblasts were serum starved for 3 h and pretreated without/with 10 µM KN-62 (left panel), or 5 µM tat-ctrl control or 5 µM tat-CN21 inhibitory peptide for 30 min followed by the addition of 50 nM insulin for 20 min as indicated. 2-Deoxyglucose uptake was measured at room temperature for 5 min. Shown are the means ± S.E. relative to basal NT cells from three independent experiments. (C) GFP-CaMKIIβ WT (GFP-CaMKII) colocalizes with α-Actinin-4 in insulin-induced ruffles. L6-GLUT4myc myoblasts were transfected with GFP-CaMKIIβ, treated with insulin as above, followed by immunostaining for endogenous α-Actinin-4. Shown are representative combined confocal images (from three experiments). Scale bar: 10 µm. (D) Endogenous CaMKII co-immunoprecipitates with α-Actinin-4. Lysates of serum-starved L6-GLUT4myc myoblasts were pretreated with GFP-CaMKIIβ, treated with insulin as above, followed by immunoprecipitation and immunoblotting (IB) with anti-α-Actinin-4 or anti-CaMKII antibodies. Shown are single representative blots from three independent experiments.
Supplementary figure 8. CaMKII is not required for insulin-stimulated GLUT4 translocation to the plasma membrane. (A) L6-GLUT4myc myoblasts pretreated with KN-62 (10 μM), vehicle (DMSO), a cell permeating peptide inhibitor of CaMKII (tat-CN21, 5 μM) or its control (tat-ctrl, 5 μM) were treated with insulin (100 nM, 20 min). Intact cells were labeled with anti-myc antibody and surface GLUT4myc was determined as in Materials and Methods. Data shown are fold increase over basal NT (non-treated) for each experimental condition ((mean ± SE, n=2). (B) Wild-type L6 myoblasts were transiently co-transfected with GLUT4-RFP and either AC3C-GFP (control) or CaMKII inhibitory peptides KIIN-GFP and AC3I-GFP. After 48-72 h, cells were treated with/without insulin for 20 min followed by surface GLUT4myc analysis in individual GFP-positive cells (> 25 cells/condition). Surface GLUT4 levels are shown as fold increase relative to AC3C-GFP basal (mean ± SE, n =2). (C) L6-GLUT4myc myoblasts were transiently transfected with GFP, GFP-CaMKIIβ (WT), constitutively active GFP-CaMKIIβ T287D, ATP binding-impaired GFP-CaMKIIβ-K43R or CaM binding-impaired GFP-CaMKIIβ-A303R. Cell surface GLUT4myc was labeled using anti-myc antibody and analyzed by measuring its fluorescent intensity in GFP-positive cells. Results are presented as fold increase relative to the value in unstimulated (basal) GFP-transfected cells (mean ± SE, n =4). (D) L6-GLUT4myc myoblasts were transfected with siNR or siCaMKIIδ (200 nM). Shown is a representative blot from 3 experiments. (E) WT L6 myoblasts transfected with siNR or siCaMKIIδ were transiently transfected with GLUT4myc-GFP. After 48 h cells were treated with/without insulin followed by surface GLUT4myc labeling in non-permeabilized cells, then permeabilized to label pThr286/7 CaMKII. SiCaMKIIδ knockdown cells with low pThr286/7 signal were selected for analysis (>30 cells/condition). Data are fold increase over basal NR (mean ± SE, n=4).
Supplementary figure 9. Total RNA was isolated from L6-GLUT4myc myoblasts (1), myotubes (2) and rat skeletal muscle (3) and used for RT-PCR detection of CaMKIIα, CaMKIIβ, CaMKIIγ and CaMKIIδ transcripts within a linear cycle period, as described in Materials and Methods. Arrowheads mark the samples of RNA isolated from L6-GLUT4myc myoblasts. The relative expression level of each isoform in myoblasts, normalized to its expression in rat skeletal muscles is indicated below the PCR bands (2.3, 1.5, 1.0, 3.4 for CaMKII α, β, γ and δ isoforms, respectively). This suggests CaMKIIδ is a predominant isoform in myoblasts.

References: