α-Actinin-4 Is Selectively Required for Insulin-induced GLUT4 Translocation

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Insulin induces GLUT4 translocation to the muscle cell surface. Using differential amino acid labeling and mass spectrometry, we observed insulin-dependent co-precipitation of actinin-4 (ACTN4) with GLUT4 (Foster, L. J., Rudich, A., Talior, I., Patel, N., Huang, X., Furtado, L. M., Bilan, P. J., Mann, M., and Klip, A. (2006) J. Proteome Res. 5, 64–75). ACTN4 links F-actin to membrane proteins, and actin dynamics are essential for GLUT4 translocation. We hypothesized that ACTN4 may contribute to insulin-regulated GLUT4 traffic. In L6 muscle cells insulin, but not platelet-derived growth factor, increased co-precipitation of ACTN4 with GLUT4. Small interfering RNA-mediated ACTN4 knockdown abolished the gain in surface-exposed GLUT4 elicited by insulin but not by platelet-derived growth factor, membrane depolarization, or mitochondrial uncoupling. In contrast, knockdown of α-actinin-1 (ACTN1) did not prevent GLUT4 translocation by insulin. GLUT4 colocalized with ACTN4 along the insulin-induced cortical actin mesh and ACTN4 knockdown prevented GLUT4-actin colocalization without impeding actin remodelling orAkt phosphorylation, maintaining GLUT4 in a tight perinuclear location. We propose that ACTN4 contributes to GLUT4 traffic, likely by tethering GLUT4 vesicles to the cortical actin cytoskeleton.

Insulin-regulated glucose transporter 4 (GLUT4) is a member of the SLC2A facilitative glucose transporter family (2) and is responsible for glucose entry into muscle and fat tissues (3–5). GLUT4 continuously cycles to/from the cell membrane through a series of endosomal compartments. In response to insulin there is a rapid increase in the steady-state level of GLUT4 at the cell surface, at the expense of intracellular pools (6–9). This process is defective in insulin resistance and type 2 diabetes (10–12). Stimuli other than insulin such as muscle contraction, depolarization, or hypoxia also increase surface GLUT4 (13–16). Whereas insulin largely increases the exocytic arm of GLUT4 cycling (17), hypoxia or membrane depolarization preferentially reduce GLUT4 endocytosis in muscle cells (16, 18, 19). Moreover, although insulin-dependent GLUT4 translocation requires dynamic remodeling of filamentous actin (20–23), the gain in surface GLUT4 elicited by platelet-derived growth factor (PDGF), depolarization, or mitochondrial uncouplers is independent of actin dynamics (24–26).

Intensive research has recently focused on identifying the individual mechanisms participating in GLUT4 traffic and the specific events regulated by insulin (27–31). Hypothesizing that GLUT4 traffic may be regulated by interaction with partner proteins, it is of fundamental and clinical interest to identify such proteins. Accordingly, we recently applied the novel SILAC (stable isotope labeling by amino acids in cell culture) approach (32) to search for proteins that associate with GLUT4 in an insulin-regulated manner (1). The study took advantage of the stable expression in L6 muscle cells of GLUT4 encoding an myc tag that faces the extracellular or luminal spaces. Immunoprecipitation via the myc antibody with endogenous partners of GLUT4 approach (32) to search for proteins that associate with GLUT4 was marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GLUT4, glucose transporter 4; SILAC, stable isotope labeling by amino acids in cell culture; ACTN, α-actinin; PDGF, platelet-derived growth factor; DNP, dinitrophenol; siRNA, small interfering RNA; siACTN4, ACTN-4-specific siRNA; siNR, non-related control siRNA; NT, non-transfected cells; Akt, protein kinase B; P13K, phosphoinositide 3-kinase; VAMP, vesicle-associated membrane protein; GFP, green fluorescent protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline.

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GLUT4 beneath the cell membrane exclusively in response to insulin.

EXPERIMENTAL PROCEDURES

Reagents, Constructs, and siRNAs—α-Phenylenediamine dihydrochloride, monoclonal anti-ACTN1 (mouse IgM, clone BM-75.2 (37)), anti-β-actin or polyclonal anti-myc antibodies, rat PDGF-BB, iron-saturated human transferrin, and LY249002 were from Sigma–Aldrich. 9E10 monoclonal antibody affinity matrix (AFC-150P) was from Covance Research Products (Berkeley, CA). Monoclonal (9E10) and polyclonal (A-14) antimyc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal ACTN4 antibody was from Alexis Biochemicals (San Diego, CA). Polyclonal anti-Akt and antiphospho Akt (serine 473) antibodies were from Cell Signaling Technology (Beverly, MA). Indocarboxyanine (Cy3)–, Cy5–, or horseradish peroxidase-conjugated goat-anti-mouse and anti-rabbit IgG antibodies or donkey anti-mouse IgM were from Jackson ImmunoResearch Laboratories (West Grove, PA). Isopropylthiogalactopyranoside, rhodamine-bound phalloidin, and Alexa 488/Alexa 647-conjugated goat anti-mouse and antirabbit IgG antibodies were from Invitrogen. d-[2-deoxy-3H]glucose and 125I-labeled transferrin were from PerkinElmer Life Sciences. siRNAs targeted against ACTN4 (siACTN4, UCA ACG AAC UGG ACU AUU AUU), ACTN1 (siACTN1, CAC UUA UCU UCG ACA AUA A), or non-related control (siNR, AUU CUA UCA UCG GGA CUU) were from Qiagen (Valencia, CA). Enhanced green fluorescent protein-tagged human ACTN4 (ACTN4-GFP) cDNA, previously described (38, 39), was a kind gift of Dr. Kazufumi Honda (National Cancer Center Research Institute, Tokyo, Japan). Enhanced green fluorescent protein-tagged C-terminal K-Ras tail (K-Ras tail-GFP) cDNA, as in Yeung et al. (40), was a kind gift of Dr. Sergio Grinstein (Hospital for Sick Children, Toronto). All cDNA constructs were prepared by Qiagen Hi-Speed Maxi-prep kits according to the manufacturer’s protocol.

Cell Culture and Transfections—L6 rat myoblasts stably expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc) and L6 wild-type myoblasts were differentiated into myotubes as described (34, 35). Post-seeding day-2 myoblasts or day-4 myotubes were non-transfected (NT) or transfected with 100 nM siACTN4, siACTN1, or siNR using calcium phosphate (CellPhect Transfection kit; GE Healthcare). siRNA-calcium phosphate precipitates were removed 12 h after addition, and cells were maintained for 72 h until experimentation. siACTN4 and siACTN1 were each effective against their targets (see “Results”). K-Ras tail-GFP or ACTN4-GFP cDNAs were, respectively, transfected by calcium phosphate as above or Lipofectamine 2000® (Invitrogen).

Cell Lysates and Immunoblotting—Cells were lysed as described (41), and equal protein samples were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad), and immunoblotted with anti-ACTN4 or anti-ACTN1 (1:2000), anti-β-actin (1:5000), anti- phospho-Ser473 Akt, phospho-Thr308 Akt, anti-phosphoextracellular signal-regulated kinase (1:1000), or anti-myc (1:1000) antibodies. Horseradish peroxidase-bound secondary antibodies were detected by Western Lightning™ chemiluminescence reagent plus (PerkinElmer Life Sciences).

GLUT4myc Immunoprecipitation—L6-GLUT4myc myoblasts seeded in 10-cm dishes and grown to confluence or differentiated into myotubes were serum-deprived for 4 h before stimulation with 100 nM insulin for 20 min, 50 ng/ml PDGF for 7 min, or 25 µM LY249002 for 20 min at 37 °C. Lysates (1.5 mg) were immunoprecipitated with 40 µl of 9E10 Monoclonal Affinity Matrix as described (1).

GST Pulldown Assay—Fragments corresponding to the GLUT4 N'-terminal tail (amino acids 1–24), large central loop (amino acids 222–287), and C'-terminal tail (476–509) were fused together and ligated into the EcoRI and XhoR sites of pGEX-2T vector (Amersham Biosciences) and called cyto-GLUT4-GST. The sequence coding for human ACTN4 was cut from the ACTN4-GFP construct and introduced into the Nde1 and Nhel sites of vector pET-27b (+) (here called ACTN4-His). The fusion proteins and GST were expressed in DH5α cells after induction with 0.3 mM isopropylthiogalactopyranoside and purified on glutathione-Sepharose (GE Healthcare) or nickel-nitrilotriacetic acid-agarose (Qiagen), respectively. Purified GST was used as negative control. Myoblasts were lysed in PBS with 1% Triton X-100 and protease inhibitors. Freshly prepared cytoGLUT4-GST and GST bound to beads were mixed with 1 mg of cell lysate or ACTN4-His, incubated overnight at 4 °C, harvested by centrifugation, and washed in PBS buffer containing Triton X-100. Bound proteins were eluted in sample buffer and visualized by immunoblotting.

Glucose Uptake and Cell-surface GLUT4myc Detection—Cells grown in 24-well plates and serum-starved for 3–5 h were treated without or with 100 nM insulin for 20 min, and 2-deoxyglucose uptake was measured as described (5) using 10 µM [2-deoxy-3H]glucose. Cell-surface GLUT4myc was detected as described (35, 42). Briefly, serum-deprived cells left untreated or treated for 7 min with 50 ng/ml PDGF or for 20 min with 100 nM insulin, 0.5 mM DNP, or 120 mM K+ at 37 °C as in Wijesekara et al. (18) and Torok et al. (25) were washed twice with ice-cold PBS, blocked 10 min with 3% (v/v) goat serum, and reacted with polyclonal anti-myc antibody (1:200) for 1 h at 4 °C. Cells were fixed for 10 min with 3% paraformaldehyde, reacted with horseradish peroxidase-bound goat anti-rabbit secondary antibody (1:2000) for 1 h at 4 °C, washed 6 times with PBS, and incubated with 1 µl o-phenylenediamine dihydrochloride reagent and allowed to develop for 20–30 min in the linear range in the dark at room temperature. The reaction was stopped with 1 ml/well of 3 N HCl. Supernatants were collected and absorbance was measured at 492 nm. Background absorbance obtained in the absence of anti-myc antibody was subtracted from all values.

Immunofluorescence Microscopy, Image Acquisition, and Analyses—After insulin treatment, cells were fixed and permeabilized in 0.1% (v/v) Triton X-100 for 3 min at 4 °C to preserve actin morphology. Labeling of actin filaments with rhodamine-bound phalloidin and antigen-specific immunostaining was as noted (43), using monoclonal anti-myc 1:100 and polyclonal anti-ACTN4 1:200 in 0.1% (w/v) bovine serum albumin in PBS, together with fluorophore-coupled secondary antibodies for 1 h at room temperature. To prevent artifac-
tual co-localization, fluorophores of maximal spectral separation were used. To reduce possible fluorophore spectra crossover, cells were imaged by multichannel scanning with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Acquisition parameters were adjusted to exclude saturation of the signal.

For rounded-up L6-GLUT4myc myoblasts, cells detached from the substratum with Ca^{2+}- and Mg^{2+}-free PBS at 37 °C were left untreated or treated with 100 nm insulin for 20 min at 37 °C on re-attachment to glass coverslips as noted. Fixed cells were processed intact for myc epitope detection or permeabilized for staining of the myc epitope, ACTN4 or actin, as above. Images were obtained with a Zeiss Axioplan 100M laser scanning confocal microscope at room temperature using a 60 × oil objective at the same gain setting otherwise. The gain detector was set using cells labeled with only secondary antibody. Rounded-up myoblasts with peripheral GLUT4 signal (>50 cells per condition) were scored “blindly,” and the number of positive cells expressed as a percentage of cells counted.

**125I-Labeled Transferrin Recycling**—Transferrin recycling was examined as noted (44). Cells were loaded with 125I-labeled transferrin (1 μg/ml) for 30 min at 37 °C and washed once each with cold medium, acidic solution (0.15 M NaCl, 0.1 M glycine, pH 3.0) and again with medium. Cells were incubated with growth medium for 2, 4, 8, or 12 min at 37 °C, and media were collected for each time point. Cells were scraped into 1 M NaOH, and protein in the media was precipitated with 20% trichloroacetic acid. Radioactivity content in the cell extract (with internalized transferrin) or media pellet (with externalized transferrin) was counted. For each time point collected in triplicate, recycled 125I-labeled transferrin was calculated as the ratio of externalized versus internalized 125I-labeled transferrin. Data were corrected for nonspecific cell-associated 125I-labeled transferrin, determined from parallel cell cultures incubated with 20 μg/ml unlabeled transferrin during loading.

**Statistical Analysis**—Results are shown as -fold values relative to the indicated control conditions in each figure. Statistical analysis was performed using Student’s t test; p < 0.05 was considered to be statistically significant.

**RESULTS**

**Insulin Specifically Enhances ACTN4 Interaction with GLUT4**—Recently we reported an interaction between ACTN4 and GLUT4 in a yeast two-hybrid screen of a 3T3-L1 adipocyte cDNA library using a hybrid protein encoding only the cytosolic regions of GLUT4 (1). ACNT4 is also expressed in skeletal muscle tissue, confirmed by immunoblot analysis of oxidative (soleus) and glycolytic (extensor digitorum longus) mouse muscles (data not shown). To further understand the functional significance of the interaction between ACTN4 and GLUT4, we first explored the specificity of insulin action on this association. In L6-GLUT4myc myotubes, ACTN4 was notably detectable in GLUT4myc immunoprecipitates in response to insulin in comparison with the basal state (Fig. 1A, top), relative to total amounts of GLUT4myc immunoprecipitated in each condition. Neither GLUT4myc nor ACTN4 was immunoprecipitated by anti-myc antibody from L6 wild-type cell lysates devoid of GLUT4myc (Fig. 1A, bottom).

Like insulin, acute stimulation with PDGF also promotes GLUT4myc externalization and glucose transport in L6 myoblasts (25). However, this response is insensitive to actin disruption by latrunculin B or to VAMP2 inactivation by tetanus toxin, hallmarks of insulin action (25, 45). Therefore, we explored whether PDGF would affect the GLUT4-ACTN4 interaction. Interestingly, PDGF did not increase the binding of ACTN4 to GLUT4myc (Fig. 1B), highlighting the specificity of insulin on this association. These experiments were tested in myoblasts, as the PDGF receptor is only expressed at the stage of the myogenic process.

Because PI3K is required for insulin-mediated recruitment of GLUT4 (46), the interaction of GLUT4myc with ACTN4 was examined in the presence of the PI3K inhibitor LY294002. L6GLUT4myc myoblasts were pretreated with LY294002 for 20 min before and during insulin stimulation. This treatment prevented the insulin-stimulated association of GLUT4myc with ACTN4 (Fig. 1C), offering additional support for the specificity of this interaction in response to insulin.

To verify that ACTN4 and GLUT4myc directly interact, as predicted from the two-hybrid analysis, we conducted pull-down assays of ACTN4 via cytoGLUT4-GST using cell extracts and pure recombinant proteins. As shown in Fig. 1D, cytoGLUT4-GST effectively pulled down endogenous ACTN4

**FIGURE 1. Insulin, but not PDGF, stimulates ACTN4 binding to GLUT4, and the two proteins interact in vitro.** A–C, serum-starved L6-GLUT4myc or L6 wild-type myotubes (A) and myoblasts (B and C) were treated with 100 nm insulin for 7 or 20 min (7 min when in parallel to PDGF), 50 ng/ml PDGF for 7 min, or 25 μM LY249002 for 20 min before and during the 20 min of incubation with insulin. Lysates were prepared and subjected to immunoprecipitation (i.p.) with anti-myc monoclonal antibody-bound beads. Samples were resolved by SDS-PAGE and immunoblotted (i.b.) with anti-ACTN4 and anti-myc polyclonal antibodies. D, L6-GLUT4myc cell lysates or purified ACTN4-His was added to cytoGLUT4-GST or GST bound to-Sepharose beads. GST pull-down extracts were resolved by SDS-PAGE and immunoblotted with anti-ACTN4 antibody. Shown are representative blots from four independent experiments each.
from cell lysates and purified, recombinant ACTN4-His. In contrast, GST alone did not pull down ACTN4 in either assay.

**ACTN4 Knockdown Reduces Insulin-stimulated Glucose Transport and Surface GLUT4 Gain**—To assess the functional relevance of ACTN4 to GLUT4 traffic, we explored the effects of silencing ACTN4 expression using a specific small interfering RNA oligonucleotide (siACTN4). Significant knockdown (60 ± 2%) of ACTN4 protein was achieved in siACTN4 (Fig. 2A) relative to NT or non-related control siRNA (siNR)-treated cells. In contrast, expression of GLUT4 or the ACTN1 isoform was unaltered. ACTN4 knockdown significantly inhibited insulin-stimulated 2-deoxyglucose uptake (1.6 ± 0.2-fold) compared with the response to the hormone in NT (2.65 ± 0.12-fold) or siNR (2.6 ± 0.04-fold) cells. In contrast, ACTN4 knockdown did not affect hexose uptake in the unstimulated state (Fig. 2B). Importantly, siACTN4 did not perturb insulin signaling at the level of Akt phosphorylation on Ser-473 (Fig. 2C) or Thr-308 (data not shown).

Insulin-stimulated translocation of GLUT4myc to the cell surface was almost obliterated by siACTN4 without any consequence on basal cell surface GLUT4myc levels (Fig. 3A). These results suggest that the residual glucose uptake response observed in siACTN4-treated cells is likely ascribed to GLUT1. In contrast to insulin, ACTN4 knockdown had no inhibitory effect on the gain in surface GLUT4myc elicited by PDGF, K⁺-mediated depolarization, or DNP-mediated mitochondrial uncoupling (Figs. 3, A and B).

**ACTN1 Knockdown Does Not Affect GLUT4 Traffic**—Because muscle cells also express ACTN1, we examined the effect of insulin on the association of this isoform with GLUT4. As shown on the Fig. 4A, insulin did not enhance the association of these proteins. Moreover, siRNA-mediated knockdown of ACTN1 (by 70%) was innocuous to basal or insulin-stimulated levels of surface GLUT4myc (Fig. 4, B and C) without affecting ACTN4 levels. Together, these results suggest that the interaction of ACTN4 with GLUT4 plays a functional and specific role in insulin-stimulated glucose uptake and GLUT4 translocation.

**ACTN4 Knockdown Does Not Alter Perinuclear GLUT4 Distribution**—In the basal state GLUT4myc predominantly assumes a perinuclear, unipolar distribution, and insulin provokes a redistribution of GLUT4 around the nuclear perimeter (47). Given that siACTN4 inhibited surface GLUT4 gain and consequent glucose uptake in response to insulin, we explored whether the basal or insulin-stimulated perinuclear GLUT4 distributions were altered. Silencing ACTN4 expression had no effect on such distribution relative to siNR-transfected cells (supplemental Fig. 1A).

**ACTN4 Knockdown Does Not Alter Transferrin Recycling**—The ACTN4-Hrs-BERP-mysin V complex (CART complex) is necessary for transferrin receptor recycling in HeLa cells (44). Therefore, we examined the effect of siACTN4 on ¹²⁵I-labeled transferrin recycling in L6-GLUT4myc cells. In contrast to the findings in HeLa cells, where ACTN4 knockdown reduced transferrin recycling, in L6-GLUT4myc cells siACTN4 did not alter this process, i.e. recycling was similar to that observed in siNR-transfected cells (supplemental Fig. 1B). Hence, the effect of siACTN4 on GLUT4 traffic cannot be ascribed to a generalized inhibition of the recycling pathway.
ACTN4 Knockdown Prevents Insulin-induced GLUT4 Co-localization with Remodeled Actin—Insulin stimulation induces a rapid reorganization of actin filaments into a cortical mesh in muscle cells, and actin dynamics is required for insulin-stimulated GLUT4 translocation in myocytes (21, 43, 48) and adipocytes (49, 50). Because ACTN4 can cross-link actin filaments and link membrane proteins to F-actin, we explored whether it might participate in actin remodeling. F-actin is detectable by rhodamine-conjugated phalloidin as stress fibers in the basal state and as dorsal ruffled structures in response to insulin (20, 48). These morphological characteristics were unaltered in siNR- or siACTN4-transfected cells (Fig. 5A). ACTN4 colocalized with the insulin-remodeled actin mesh in siNR-treated cells and was virtually absent from these sites in siACTN4-transfected cells (Fig. 5A). Thus, ACTN4 knockdown is inconsequential on insulin-induced actin remodeling. To further confirm these observations, actin remodeling was also examined in rounded-up myoblasts. In this cellular configuration, actin remodels in response to insulin to form peripheral arborizations (23). ACTN4 knockdown had no effect on this response (Fig. 5B). This figure also shows that ACTN4 concentrates at the cell periphery, although some intracellular localization is also evident. In insulin-stimulated cells ACTN4 is found along the remodeled cortical actin.

We next examined the consequence of ACTN4 knockdown on GLUT4 localization vis-à-vis the actin mesh. As shown earlier, GLUT4/myc does not co-localize with actin filaments in unstimulated cells. Upon insulin stimulation, GLUT4/myc abundantly colocalizes with the insulin-remodeled actin mesh (43) along with ACTN4 (1). Strikingly, following ACTN4 knockdown GLUT4/myc no longer co-localized with the insulin-triggered actin mesh despite the visually normal actin remodeling (Fig. 5A). In contrast, siNR-transfected cells displayed the typical co-localization of GLUT4/myc with the cortical actin mesh. These results suggest that ACTN4 is required for the physical positioning of GLUT4 vis-à-vis the remodeled actin.

Peripheral, Submembrane GLUT4 Accumulation—GLUT4 externalization at the cell surface requires the movement of GLUT4 vesicles to the plasma membrane, where they subsequently fuse to expose regions of the transporter to the extracellular medium. Current models based on work in 3T3-L1 adipocytes debate whether GLUT4 vesicles gain access to the cell periphery in response to the hormone or whether they continuously approach the membrane where insulin signals promote docking and fusion (6, 8, 29, 51). There is no equivalent analysis for GLUT4 in muscle cells. Here, we took advantage of the ease of detecting GLUT4/myc in the perinuclear and peripheral regions in rounded-up myoblasts (52) to explore the consequence of ACTN4 knockdown on GLUT4/myc localization. While 80 ± 1% of siNR-treated cells showed insulin-dependent gain in surface-exposed GLUT4/myc in non-permeabilized cells, only 26.5 ± 2.9% of siACTN4-treated cells displayed such gain (Fig. 6A). These results are in good agreement with the observations made with adhered monolayers of L6 myocytes shown in Fig. 3. Interestingly, under the same conditions of ACTN4 knockdown and insulin stimulation, cell permeabilization revealed that GLUT4/myc was largely retained in the perinuclear region (Fig. 6B). Although insulin-stimulated, only 25 ± 5% of siACTN4-treated cells displayed peripheral GLUT4/myc compared with 73 ± 6% of siNR-treated cells. This 25% matches the proportion of cells with GLUT4/myc exposed at the surface shown in Fig. 6A. Hence, ACTN4 knockdown does not allow any submembrane accumulation of GLUT4/myc.

The specificity of siACTN4 was demonstrated through rescue experiments in which full-length, human ACTN4-GFP was expressed into siACTN4-treated cells. Under these conditions, recovery of GLUT4/myc arrival and fusion with the plasma membrane was observed in 93 ± 10% of cells (Fig. 6C). Importantly, there was no effect of full-length human ACTN4-GFP in unstimulated cells, demonstrating that ACTN4 expression does not suffice to mobilize the transporter; rather, it is an essential component for GLUT4 mobilization upon insulin signaling.

To further document the absence of GLUT4/myc at the cell periphery caused by ACTN4 knockdown, siACTN4 was cotransfected along with the plasma membrane marker K-Ras tail-GFP (40, 53). siNR-treated cells formed GLUT4/myc peripheral rims in response to insulin stimulation, which colocalized with K-Ras tail-GFP. In contrast, there was no visible colocalization of GLUT4/myc with K-Ras tail-GFP in cells transfected with siACTN4 after insulin stimulation (supplemental Fig. 2). The above results illustrate that ACTN4 knockdown results in intracellular/perinuclear retention of

**Figure 4.** ACTN1 knockdown does not alter GLUT4 traffic. A, lysates of serum-starved L6-GLUT4/myc or L6 wild-type myotubes were treated without or with 100 nm insulin for 20 min at 37 °C and immunoprecipitated (i.p.) with anti-myc antibody-bound beads (intraperitoneal myc). i.b., immunoblot. B and C, L6-GLUT4/myc myotubes were either NT or transfected with 100 nm siNR or siACTN1. A and B, samples resolved by SDS-PAGE were immunoblotted with anti-ACTN4 or anti-ACTN1, anti-myc, or β-actin antibodies. Shown are representative blots from four independent experiments. C, serum-depleted cells were stimulated with 100 nm insulin for 20 min, and surface-myc-tagged GLUT4 density was quantified using the antibody-coupled colorimetric assay. Shown are the means ± S.E. relative to basal NT cells from four independent experiments, *, p ≤ 0.05.


**DISCUSSION**

The gain in glucose transporter GLUT4 at the muscle cell surface is central for the ability of the body to clear dietary glucose from the circulation. Impairments in this process are hallmarks of insulin resistance, leading to type 2 diabetes. Because more than 150 million individuals worldwide suffer from this disorder (54) and insulin resistance is prevalent in obese non-diabetic individuals, mapping the molecular elements of GLUT4 traffic is of major importance. In search for proteins that might regulate this process, we performed a differential proteomic screen using SILAC to identify insulin-induced changes in the protein profile of GLUT4-coprecipitating proteins. ACTN4 emerged as the protein with the most prominent increased association with GLUT4 (1). Here we describe that ACTN4 is essential for the insulin-induced gain in surface GLUT4 in muscle cells, specifically for the redistribution of the transporter to the cell periphery.

ACTN4 belongs to a family of actin-interacting proteins that includes spectrin, dystrophin, and utrophin (33, 55). ACTNs 1 and 4 are ubiquitously expressed, whereas ACTNs 2 and 3 are muscle-specific, cross-linking sarcomeric actin filaments in the Z line. Instead, ACTNs 1 and 4 bind actin filaments to the cortical cytoskeleton and interact with cytoskeletal- and membrane-associated proteins. Both encode an N-terminal actin binding region with a phosphatidylinositol 4,5-diphosphate binding site (37, 56), a central rod of four spectrin-like repeats and a calmodulin-like domain (33, 37). ACTN1 is primarily found in adhesion plaques and junctions, where it cross-links actin filaments in a Ca2+-sensitive fashion and links stress fibers to the membrane via talin, integrin, and vinculin (33, 57, 58). In contrast, ACTN4 is absent from adhesion plaques or junctions (37), and its interaction with actin is Ca2+-insensitive (59–61). ACTN4 is found at dorsal surface ruffles in macrophages (62) and plasma membranes in podocytes (63) and participates in cell motility, exosome formation (64), and transferrin receptor recycling (44).

These diverse functions have sparked the search for ACTN4 binding proteins, and a discrete number of partners has been described: nitric-oxide synthase (65), the Ring finger BERP (61), Hrs (44), Na/H exchanger 3 (66), the p85 subunit of PI3K and Akt1 (67), the tight junction protein MAGI-1 (68), the Rab5-GAP RN-tre (69), and as mentioned above, GLUT4. This latter interaction appears to be direct, as recombinant ACTN4 bound to a construct encoding the cytosolic regions of GLUT4 (Fig. 1D).

Strikingly, the coprecipitation of ACTN4 with full-length GLUT4 from muscle cells was insulin-dependent, and given the participation of the actin cytoskeleton in insulin-dependent GLUT4 traffic, we hypothesized that ACTN4 may participate in insulin-dependent GLUT4 gain in surface GLUT4. The...
enhanced coprecipitation was specific for insulin and did not occur with PDGF. This is noteworthy as PDGF also binds to a tyrosine kinase-bearing receptor and enhances surface GLUT4 through PI3K-Akt-AS160 signaling. However, the response to PDGF is insensitive to cytoskeletal disruption by cytochalasin D or latrunculin B, drugs that abort the insulin response of GLUT4 (25, 69). Moreover, PDGF recruits GLUT4 from the recycling endosome and involves VAMP7, whereas insulin recruits from a specialized compartment functionally characterized by VAMP2 (70, 71).

The central observation of this study is that ACTN4 knockdown prevents the insulin-dependent gain in surface GLUT4 in myoblasts and myotubes but does not affect the gain in surface GLUT4 brought about by PDGF or by agents that mimic the stress or contraction pathways, such as K<sup+</sup>-induced depolarization and mitochondrial uncoupling via DNP. This is interesting as K<sup+</sup>- and DNP largely act by reducing GLUT4 endocytosis (16, 18, 25), and additionally, the vesicles that undergo exocytosis in response to PDGF or insulin differ (25). Thus, ACTN4 selectively participates in the insulin-regulated exocytosis of GLUT4. Importantly, ACTN1 knockdown did not affect the insulin-dependent gain in surface GLUT4. Hence, ACTN4 displays functional specificity compared with its most similar isoform. The results also suggest that ACTN1 cannot overcome the loss of ACTN4 toward GLUT4 traffic.

The preventive effect of ACTN4 knockdown on insulin-dependent gain in surface GLUT4 was overcome by concomitant expression of human ACTN4-GFP, underscoring the specificity of the knockdown approach used herein. Because ACTN4 knockdown did not affect the insulin-dependent gain in surface GLUT4, Hence, ACTN4 displays functional specificity compared with its most similar isoform. The results also suggest that ACTN1 cannot overcome the loss of ACTN4 toward GLUT4 traffic.

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ACTN4 knockdown, and such levels are maintained by continuous GLUT4 recycling to the membrane with a half-time of about 2 h (17).

The most pressing challenge is to define the mechanism of action of ACTN4 in insulin-stimulated GLUT4 traffic to the membrane. ACTN4 knockdown did not alter insulin signaling to Akt nor insulin-induced actin reorganization. This is important, as ACTN4 is found along the remodeled actin mesh upon insulin stimulation. Hence, actin remodeling relocates ACTN4, but ACTN4 does not govern insulin-induced actin reorganization.

A key finding is that ACTN4 knockdown precluded the insulin-dependent relocation of GLUT4 into the region of remodeled actin. This may explain why ACTN4 knockdown prevents the gain in surface GLUT4, as actin dynamics is required for such gain and normally GLUT4 gathers in the reorganized actin region (but not along actin stress fibers before insulin stimulation). Thus, ACTN4 may bridge GLUT4 vesicles to the remodeled F-actin at the cell periphery in an insulin-regulated manner. Analysis of rounded-up myoblasts sheds further light on ACTN4 function on GLUT4 localization. In this cellular configuration, ACTN4 is largely at the cell periphery and to a lesser extent dispersed within the cytoplasmic space (Fig. 5B). Conversely, ACTN4 is mostly at a perinuclear pole in unstimulated cells. Insulin changed the distribution of GLUT4 causing a clear increase at the cell periphery (including its full insertion into the cell membrane) and throughout the cytosol but had no major effect on ACTN4 localization. Significantly, ACTN4 knockdown prevented the gain in GLUT4 beneath the cell membrane (Fig. 6B). This differs from the mode of action of inhibitors of PI3K, which precluded insulin-dependent GLUT4 externalization (fusion with the membrane) but allowed partial accumulation beneath the membrane (72–74). Accordingly, under conditions of PI3K inhibition, GLUT4myc and ACTN4 were found near the periphery of insulin-stimulated cells (results not shown). However, the two proteins were unable to interact with each other (Fig. 1C).

How does ACTN4 contribute to GLUT4 availability at the cell periphery? We offer the following working models that incorporate the results of the present study along with current models of GLUT4 traffic. (a) Insulin signals may allow GLUT4 vesicle release from perinuclear retaining mechanisms, mobilization on microtubules, retention by ACTN4 at the cell periphery along the remodeled actin mesh, and further promote GLUT4 vesicle docking/fusion with the membrane through SNARE (soluble N-ethylmaleimide factor attachment protein receptors) proteins. According to this model, ACTN4 knockdown would abate GLUT4 vesicle tethering at the cell periphery, thereby also precluding fusion with the membrane. Interestingly, a similar anchoring role has been suggested for ACTN4-regulating inducible nitric-oxide synthase interaction with the cortical cytoskeleton in macropages (65). (b) Alternatively, GLUT4 vesicles may continuously arrive at the periphery along microtubules, only to interact with ACTN4 after insulin stimulation. This would require that the hormone facilitates a mechanism that increases the presentation of ACTN4 to GLUT4 in order to account for the insulin-dependent co-precipitation of these proteins. The insulin-induced, PI3K-dependent actin remodeling could fulfill this role. In either model, ACTN4 and GLUT4 would come in contact only at the cell periphery, as suggested from the preferential localization of ACTN4.

Whether arrival at the cell periphery is insulin-dependent (model a) or constitutive (model b), PI3K signaling could mediate transfer of vesicles from microtubules to cortical actin. Inhibition of PI3K would “freeze” GLUT4 vesicles at the cell periphery before transfer, explaining the accumulation of GLUT4 in that region and the prevention of the GLUT4–ACTN4 interaction. In contrast, ACTN4 knockdown allows PI3K-dependent signaling toward Akt to proceed so release of GLUT4 vesicles from microtubules would still occur. However, in this case the vesicles would not be able to hook onto the actin mesh lacking ACTN4. Live cell analysis of GLUT4 and ACTN4 distribution will be required to determine the dynamics of their interaction.

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