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Myosin IIA participates in docking of Glut4 storage vesicles with the plasma membrane in 3T3-L1 adipocytes

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ABSTRACT

In adipocytes and myocytes, insulin stimulation translocates glucose transporter 4 (Glut4) storage vesicles (GSVs) from their intracellular storage sites to the plasma membrane (PM) where they dock with the PM. Then, Glut4 is inserted into the PM and initiates glucose uptake into these cells. Previous studies using chemical inhibitors demonstrated that myosin II participates in fusion of GSVs and the PM and increase in the intrinsic activity of Glut4. In this study, the effect of myosin IIA on GSV trafficking was examined by knocking down myosin IIA expression. Myosin IIA knockdown decreased both glucose uptake and exposures of myc-tagged Glut4 to the cell surface in insulin-stimulated cells, but did not affect insulin signal transduction. Interestingly, myosin IIA knockdown failed to decrease insulin-dependent trafficking of Glut4 to the PM. Moreover, in myosin IIA knockdown cells, insulin-stimulated binding of GSV SNARE protein, vesicle-associated membrane protein 2 (VAMP2) to PM SNARE protein, syntaxin 4 was inhibited. These data suggest that myosin IIA plays a role in insulin-stimulated docking of GSVs to the PM in 3T3-L1 adipocytes through SNARE complex formation.

Introduction

Insulin maintains glucose homeostasis by enhancing glucose transport into muscle and adipose tissues, which is a process mediated by recruitment of Glut4 to the cell surface, where these vesicles are tethered, docked and fused with the plasma membrane (PM) [1]. One class of proteins that function in this docking/fusion step are the SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins) [2] including vesicle-SNARE (v-SNARE, such as synaptobrevin2/vesicle-associated membrane protein 2 (VAMP2) and target-SNARE (t-SNARE, such as syntaxin 4 and synaptosome associated protein 23 (SNAP23) [3,4]. The SNARE core, once formed, is extremely energetically stable, and thus the timing of its formation and subsequent dissociation is likely regulated by SNARE-associated proteins, such as Munc-18c, tomosyn, synip and pantophysin [4].

It has been reported that insulin-stimulated Glut4 trafficking and glucose uptake through Glut4 involve actin cytoskeleton [5–8] and myosin family proteins, a molecular motor that is responsible for actin-based motility [9–13]. Recently, Bose et al. have shown that Myo1c promotes the fusion of Glut4 storage vesicles with the PM [8].

Myosin II, a conventional two-headed myosin, was first identified in muscle. In non-muscle cells, myosin II has diverse functions including cytoplasmic contractility [14], cytokinesis [15], capping of cell-surface components [16], polarization of cell locomotion [17], neurite outgrowth [18] and regulation of the cytoskeletal reorganization that is required for vesicle fusion with the PM [12,19]. Non-muscle myosin IIA and myosin IIB [20–22] are expressed almost ubiquitously in higher organisms [23] including in 3T3-L1 adipocytes [10]. Inhibition of myosin II activity by blebbistatin, a myosin II family inhibitor, was reported to impair glucose uptake but not Glut4 translocation to the PM in 3T3-L1 adipocytes [10]. Recently, Fulcher et al. showed that inhibition of myosin II activity by ML7, a myosin light chain kinase inhibitor, impairs vesicle fusion with the PM and Glut4 intrinsic activity [9].

The molecular mechanism by which insulin stimulates the translocation, tethering, docking and fusion of Glut4 to the cell surface has been extensively studied. Still, the specific molecules linked to these individual steps are largely unknown. The aim of

Abbreviation: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

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the present study was to specify the role of myosin II in the distal events of Glut4 storage vesicles. By knocking down the expression of myosin IIA, we successfully demonstrate a myosin IIA-mediated process of GSVs docked to the PM through an increased binding of VAMP2, the v-SNARE protein on GSVs, to syntaxin 4 the t-SNARE protein on the PM.

Materials and methods

Materials. Calf serum (CS), fetal bovine serum (FBS), penicillin/streptomycin and 0.25% trypsin–EDTA were purchased from Life Technologies (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline without Mg^{2+} or Ca^{2+} (D-PBS), dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma–Aldrich (St. Louis, MO). Insulin was purchased from Wako (Osaka, Japan), and troglitazone was from Cayman Chemical (Ann Arbor, MI). pcDNA3-myc-Glut4 construct (myc-Glut4) containing a myc epitope tag in the first exofacial loop of Glut4 was kindly provided by Dr. Shuichi Okada (Gunma University, Japan). Enhanced chemiluminescence (ECL) detection and bicinchoninic acid (BCA) protein assay kits were purchased from Thermo Scientific (Rockford, IL), and Streptavidin–HRP was from Invitrogen (Carlsbad, CA). siGENOME SMARTpool Mouse MYH9 (M-040013-00-01) for myosin IIA knockdown (siMyosin IIA)and siGENOME non-Targeting siRNA Pool #2 (D-001206-13-20as a control (siControl) were obtained from Thermo Scientific. All other chemicals were of analytical grade.

Antibodies. Anti-VAMP2 antibody and anti-syntaxin 4 antibody were purchased from Synaptic Systems (Goettingen, Germany). Anti-IRAP antibody was a generous gift from Dr. Hashiramoto (Kawasaki Medical College, Japan). Anti-Glut1 antibody was purchased from Biogenesis (Hackensack, NJ). Anti-myosin IIA antibody was from Sigma–Aldrich (St. Louis, MO). Anti-myc (9E10) antibody, anti-IRβ, anti-IRS-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti β-actin antibody was purchased from Cell Signaling Technology. Anti-Glut4 antibody was raised and purified as described before [24]. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from GE Healthcare (Piscataway, NJ).

Cell culture. 3T3-L1 fibroblasts were induced to differentiate as described previously with minor modification [25]. Briefly, 3T3-L1 fibroblasts were cultured in 15 cm dishes with DMEM containing 4.5 g/l glucose, L-glutamine and 10% CS. Two days after confluence, cells were induced to differentiate by changing media to DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, 1.7 μM insulin, and 1 μM troglitazone. After 48 h of induction, medium was changed to DMEM supplemented with 10% FBS, and cells were fed with fresh medium every other day thereafter. Electroporation was conducted 8 days after inducing differentiation, when >90% of cells expressed the adipocyte phenotype.

Electroporation into 3T3-L1 adipocytes. This protocol was performed as described by Shuichi Okada et al. [26]. Briefly, differentiated 3T3-L1 adipocytes were washed with PBS, dislodged by incubation with trypsin–EDTA and washed twice with D-PBS by centrifugation at 900 rpm for 3 min at room temperature. Adipocytes were suspended again in D-PBS to an approximate concentration of 1.0×10^7 cell/500 μl and pipetted into a cuvette with a 0.4 cm electrode gap with either 2 nM siMyosin IIA or siControl (and with or without 400 μg of pcDNA3-myc-Glut4 for colorimetric assay, respectively). This mixture was charged with 950 μF capacitance at 0.16 kV in a Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA). Cells were then immediately diluted with 1 ml of DMEM, transferred to a tube containing warmed DMEM supplemented with 10% FBS and were incubated at RT (room temperature). After 10 min of incubation, cells were plated onto a 24-well plate for colorimetric assay and glucose uptake, a 6-well plate for Western blot analysis, or a 10 cm dish for immunoprecipitation. Approximately 12 h after electroporation, cells were fed with fresh DMEM supplemented with 10% FBS and kept incubated another 36 h. Then, cells were serum-starved for at least 4 h before experiments were performed.

Western blot analysis. After treatment of cells as described in the figure legends, they were rinsed three times with ice-cold phosphate saline buffer (PBS) and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 μM aprotinin, 10 μM leupeptin, 0.1 μM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 20 mM beta-glycerophosphate and 1 mM sodium orthovanadate, pH 7.4). Cell lysates were obtained by centrifugation at 13000 rpm at 4 °C for 20 min, and protein content was determined for each sample by the bicinchoninic acid (BCA) method. Equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Immunoblotting was performed with an ECL system according to the manufacturer's instructions. Densitometric analysis was performed using Image J 1.38 × software (NIH, Bethesda, MD).

Glucose uptake assay. Experiments were carried out as described previously [10] with little alteration. Briefly, cells were serum-starved for 4 h with serum-free DMEM and then cells were treated or not with 100 nM insulin for 15 min following a 30 min preincubation in Krebs Ringer Phosphate buffer (KRP) (0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 12.5 mM HEPES, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 120 mM NaCl, 6 mM KCl, pH 7.4). Glucose uptake was measured by incubation with 1 mM 2-deoxy-D-glucose containing 1 μCi/ml [^{3}H]-2-deoxy-D-glucose for 4 min at 37 °C. Cells were solubilized with PBS containing 0.1% Triton X-100, and the radioactivity was detected by a liquid scintillation counter LSC-6100 (Aloka, Tokyo, Japan). The background count was measured in the presence of 1 μl of 120 mM phloretin/well, which was subtracted from each count.

Immunoprecipitation. 3T3-L1 adipocytes electroporated with siRNA were serum-starved for 4 h and treated with or without 100 nM insulin for 15 min. Cell lysates were prepared as described in *Western blot analysis*. About 1 μg VAMP2 antibody was added to the cell lysate containing 2 mg protein. They were kept at 4 °C overnight with constant rotation. Then, 50 μl of protein A plus Sepharose beads was added to the mixture, and was incubated for 3 h at 4 °C. Subsequently, the immunoprecipitates were washed three times with RIPA buffer and subjected to SDS–PAGE.

Colorimetric assay for detecting myc-Glut4 proteins in the plasma membrane. 3T3-L1 adipocytes electroporated with 2 nM of siMyosin IIA or siControl plus 400 μg myc-Glut4 were reseeded onto a 24-well plate. After 48 h, wells were washed twice with serum-free DMEM at 37 °C and incubated with DMEM containing 0.2% BSA (bovine serum albumin) for 4 h. Then, cells were washed twice with KRP buffer at RT, incubated with KRP buffer for 30 min at 37 °C, and were either left untreated or treated with 100 nM insulin for 30 min at 37 °C. After treatment, cells were kept on ice and washed with ice-cold PBS, followed by fixation with 3.7% formaldehyde in PBS for 5 min. After several washes with PBS, cells were incubated with PBS containing 0.1 N glycine for 15 min at RT to block nonspecific binding of the antibodies. Cells were incubated with anti-myc antibody for 2 h at RT, followed by anti-mouse HRP-conjugated antibody in KRP with 0.1% BSA for 1 h. The intensity of the ECL (enhanced chemiluminescence) reaction was determined using an Infinite F®500 reader (TECAN Männedorf, Switzerland).

Immunofluorescence. After treating cells as indicated in each figure legend, cells were fixed with 3.7% formaldehyde in PBS for 15 min and immunostained with anti-Glut4 antibody and Cy2-

conjugated secondary antibody. Images were taken on a Leica TSC-SP2 confocal microscope (Wetzlar, Germany).

Statistical analysis. P values were calculated using *unpaired Student's t-test*, and p values <0.05 were considered as significant differences.

Results and discussion

Knockdown of myosin IIA expression with siRNA does not affect insulin signal transduction, but attenuates glucose uptake

Two previous studies by others have shown that pharmacological inhibition of myosin II activity by either blebbistatin or ML7, blocks the insulin-stimulated-glucose uptake by inhibiting Glut4 incorporation into the PM and Glut4 intrinsic activity, but not by impairing insulin signal transduction and Glut4 translocation [9,10]. They also reported that myosin IIA, but not IIB, is responsible for these insulin-dependent events, since myosin IIA is also translocated to the PM by insulin stimulation and binds Glut4. In this study, to specify the role of myosin IIA for Glut4 trafficking, gene silencing was used in 3T3-L1 adipocytes, which was established and commonly utilized recently [27]. As shown in Fig. 1A, approximately 75% reduction in the amount of myosin IIA did not influence the amount of signaling molecules essential for insulin signal transduction, such as insulin receptor β subunit (IRβ), insulin receptor substrate 1 (IRS-1) and Akt, and the amount of molecules involved in Glut4 trafficking, such as insulin-responsive amino peptidase (IRAP), glucose transporter 1 (Glut1), Glut4, syntaxin 4 and VAMP2 after quantification and normalization by β-actin expression (data not shown). Insulin-stimulated Akt phosphorylation was unaffected by myosin IIA ablation (Fig. 1B). However, knockdown of myosin IIA significantly reduced basal and insulin-stimulated glucose uptake compared to a siControl (Fig. 2).

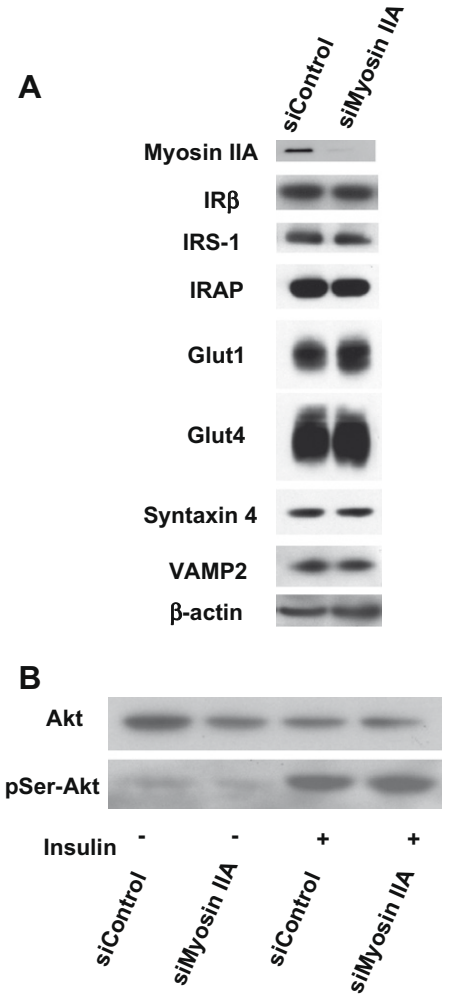

Fig. 1. Knockdown of myosin IIA did not affect insulin signal transduction. Cells were electroporated with either siControl or siMyosin IIA as described in *Materials and methods*. (A) Total cell lysates were immunoblotted with anti- myosin IIA, anti-IRβ, anti-IRS-1, anti-IRAP, anti-Glut4, anti-Glut1, anti-syntaxin 4, anti-VAMP2 or anti-β-actin antibody. (B) Cells were serum-starved and were left untreated (−) or treated with 100 nM insulin (+) for 10 min. Cell lysates were immunoblotted with either anti-Akt or anti-phosphoserine Akt antibody. Representative results out of four independent experiments are presented.

Myosin IIA inhibits docking of GSVs to the PM without any effect on Glut4 translocation

In order to investigate the mechanism of attenuated glucose uptake by myosin IIA knockdown insulin-stimulated Glut4 translocation to the PM was evaluated. Both 3T3-L1 adipocytes electroporated with siControl and siMyosin IIA similarly exhibited localization of Glut4 at the PM rim (Fig. 3A). Thus, it is unlikely that myosin IIA knockdown inhibits translocation of GSVs. It has been reported that, following translocation of GSVs to the PM, GSVs are tethered, docked, and fuse with the PM to facilitate glucose uptake through Glut4 [28]. To elucidate the mechanism of attenuated glucose transport by myosin IIA knockdown, we next evaluated insertion of Glut4 into the plasma membrane, which requires GSVs successfully tethered and docked to, and fused with the PM. To this end, insulin-stimulated surface exposure of myc-tagged Glut4, which has been known to exhibit the same kinetics as endogenous Glut4 [29] was monitored in cells with or without myosin IIA knockdown.

As shown in Fig. 3B, surface exposure of myc-tagged Glut4 was significantly reduced by myosin IIA knockdown. Thus, myosin IIA knockdown apparently inhibited insulin-stimulated insertion of Glut4 into the PM that requires a sequence of events consisting of tethering, docking and membrane fusion.

It has been reported that SNARE complex formation between syntaxin 4, VAMP2 and SNAP23 confers the molecular basis for

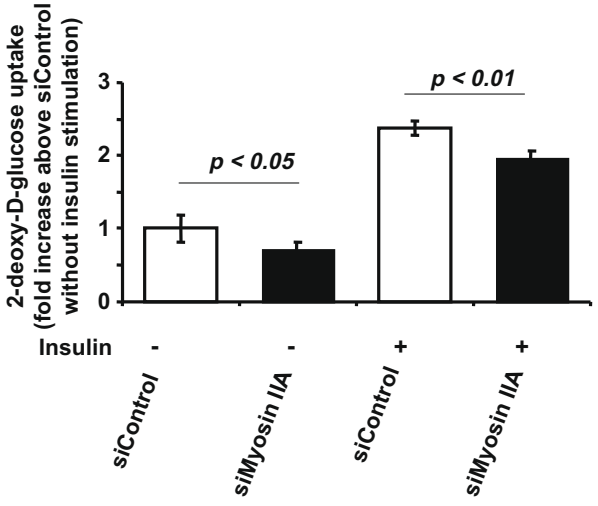

Fig. 2. Knockdown of myosin IIA decreased insulin-stimulated glucose uptake. Cells were electroporated with either siControl or siMyosin IIA and glucose uptake were measured as described in *Materials and methods*. Results from four independent experiments were quantified, and the fold-increase from siControl without insulin is shown. Data shown are means ± SEM.

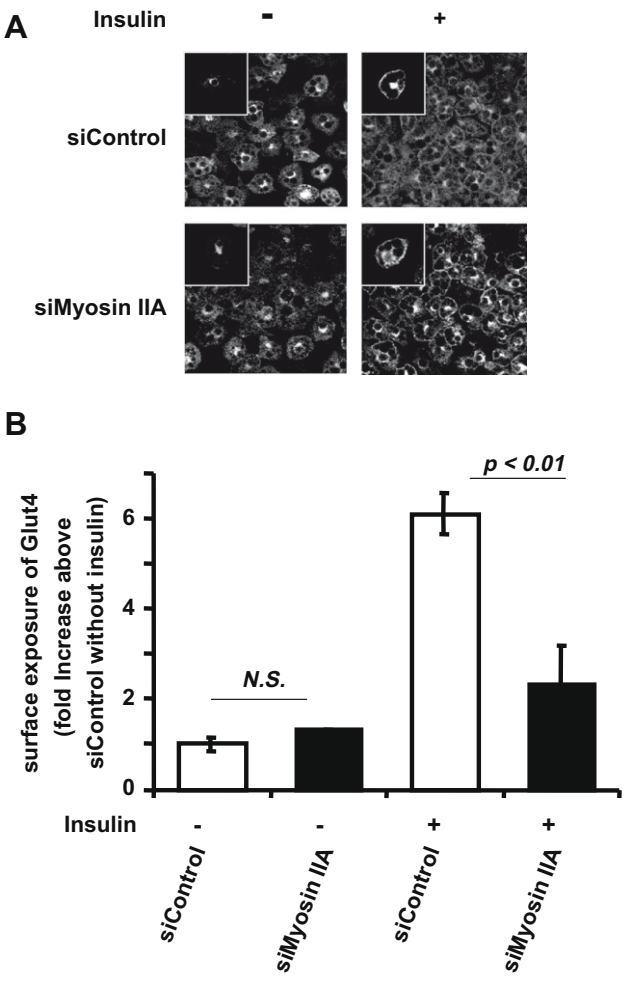

Fig. 3. Knockdown of myosin IIA inhibited Glut4 insertion into the PM, but not translocation of Glut4 to the PM. Cells were electroporated with either siControl or siMyosin IIA (A), together with pcDNA3-myc-Glut4 (B), as described in *Materials and methods* section. Cells were serum-starved and were either left untreated (−) or treated with 100 nM insulin (+) for 30 min. (A) Cells were immunostained for Glut4. Representative image out of four independent experiments are shown. (B) The amount of myc-tag exposed to the extracellular surface was quantified as described in *Materials and methods*. Results from three independent experiments were quantified, and the fold-increase from siControl without insulin is shown (B). Data shown are means ± SEM. *N.S.*, not significant.

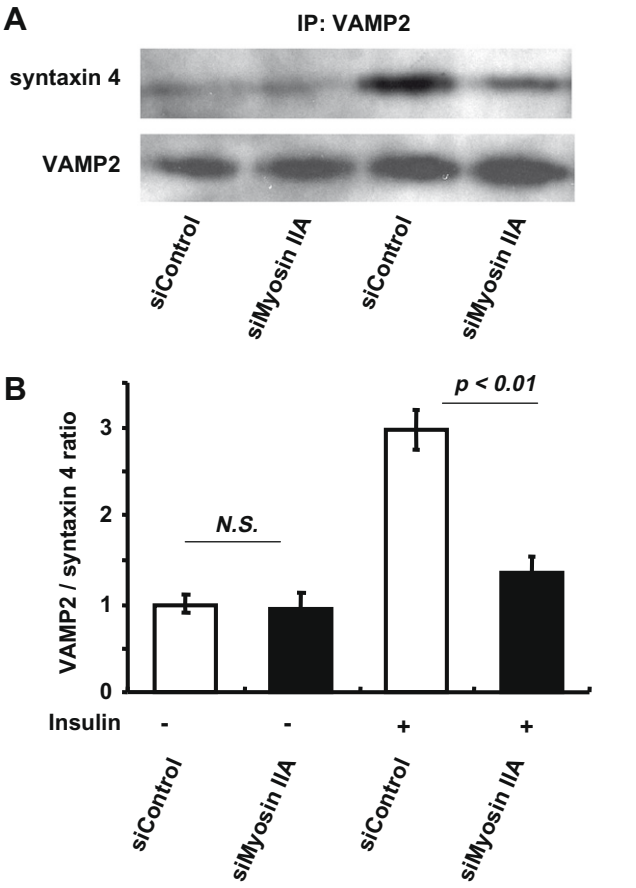

Fig. 4. Knockdown of myosin IIA inhibited SNARE complex formation. Cells were electroporated with either siControl or siMyosin IIA as described in *Materials and methods*. Cells were then serum-starved and were either left untreated (−) or treated with 100 nM insulin (+) for 10 min. Cell lysates were immunoprecipitated with anti-VAMP2 antibody and immunoblotted with either anti-syntaxin 4 or anti-VAMP2 antibody. Representative result from three independent experiments is shown in the upper panel. Results from three independent experiments were also quantified and the fold-increase from siControl without insulin is shown in the lower panel. Data shown are means ± SEM. *N.S.*, not significant.

insulin-stimulated docking of GSVs to the PM [30]. Since insulin-stimulated Glut4 localization at the PM was evident even in myosin IIA knockdown cells (Fig. 3A), we postulated that myosin IIA may play a role in Glut4 insertion into the PM after GSVs are tethered. Thus, we investigated the effect of myosin IIA knockdown on SNARE complex formation between GSVs and the PM. As shown in Fig. 4, in myosin IIA knockdown cells, insulin-dependent interaction between VAMP2 and syntaxin 4 was significantly reduced. Therefore, myosin IIA knockdown inhibited SNARE complex and docking of GSVs to the PM, which accounts for a loss of Glut4 insertion into the PM (Fig. 3B).

In this study, myosin IIA expression was knocked down to investigate the role of myosin IIA in insulin-dependent Glut4-mediated glucose uptake more specifically and more in detail. Knockdown of myosin IIA expression reduced insulin-stimulated glucose uptake (Fig. 2), but it neither decreased the expression level of proteins essential for insulin signal transduction and Glut4 translocation (Fig. 1A), nor impaired insulin signal transduction (Fig. 1B). However, myosin IIA knockdown abolished insulin-stimulated association between VAMP2 and syntaxin 4 (Fig. 4), which effectively inhibited insertion of Glut4 into the PM (Fig. 3B). These results suggest that myosin IIA is involved in insulin-stimulated docking of GSVs to the PM.

Myosin IIA knockdown, despite its remarkable inhibitory effect on GSVs docking to the plasma membrane, insulin-stimulated glucose uptake was partially inhibited. We speculate the cause of this discrepancy may be due to another role of myosin IIA on Glut4, which is an activation of its intrinsic glucose transport ability [9]. A 50% decrease in myosin IIA expression has no effect on glucose uptake (data not shown). Thus, even when more than 75% of myosin IIA expression was ablated, a small fraction of myosin IIA could be activate Glut4 that managed to get to the cell surface. Another possibility to explain this discrepancy is a contribution of Glut1 to insulin-stimulated glucose uptake. In fact, it was reported that Glut1 in 3T3-L1 adipocytes is expressed three times more abundantly than Glut4 and translocates to the PM in response to insulin [31,32]. Further study should be directed to answer the question.

In summary, our study has revealed a novel function for myosin IIA in involving insulin-stimulated membrane docking in 3T3-L1 adipocytes with the binding of v-SNARE, VAMP2, to t-SNARE syntaxin 4. The results presented here add to a growing list of factors involved in promoting docking events of GSVs to the PM and may provide further understanding into the cellular basis of impaired insulin sensitivity observed in obesity and diabetes.

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