**Development of a novel GLUT4 translocation assay for identifying potential novel therapeutic targets for insulin sensitization**

Franklin LIU*,†, Qing DALLAS-YANG*,†, Gino CASTRIOTA*,†, Paul FISCHER†, Francesca SANTINI‡, Marc FERRER‡, Jing LI†, Taro E. AKIYAMA*, Joel P. BERGER*, Bei B. ZHANG* and Guoqiang JIANG*‡


GLUT4 (glucose transporter 4) plays important roles in glucose homeostasis in vivo. GLUT4 expression and function are diminished in diabetic human and animal subjects. The goal of the present study is to develop a cell-based assay for identifying negative regulators of GLUT4 translocation as potential targets for the treatment of Type 2 diabetes. Traditional GLUT4 translocation assays performed in differentiated myocytes or adipocytes are difficult to perform, particularly in HTS (high-throughput screening) mode. In the present study, we stably co-expressed c-Myc and eGFP (enhanced GFP (green fluorescent protein)) dual-tagged recombinant GLUT4 with recombinant IRS1 (insulin receptor substrate 1) in HEK-293 cells (human embryonic kidney cells) (HEK-293. IRS1.GLUT4 cells). Insulin treatment stimulated both glucose uptake and GLUT4 translocation in these cells. GLUT4 translocation is quantified by a TRF (time-resolved fluorescence) assay in a 96-well HTS format. TRF assays confirmed insulin-stimulated GLUT4 translocation, which can be inhibited by P13K (phosphoinositide 3-kinase) or Akt (also called PKB (protein kinase B)) inhibitors. Treatment with palmitate increased GLUT4 phosphorylation and reduced insulin-stimulated Akt phosphorylation and GLUT4 translocation, indicating insulin resistance. Knockdown of PTEN (phosphatase and tensin homologue deleted on chromosome 10) and PTP1B (protein tyrosine phosphatase 1B) gene expression by siRNA (small interfering RNA) treatment significantly increased GLUT4 translocation only in cells treated with palmitate but not in untreated cells. Similar results were obtained on treatment with siRNA of JNK1 (c-Jun N-terminal kinase 1), S6K1 (ribosomal protein S6 kinase, 70 kDa, polypeptide 1) and PKCβ (protein kinase C β). In summary, we have established and validated a novel GLUT4 translocation assay that is optimal for identifying negative regulators of GLUT4 translocation. In combination with more physiologically relevant secondary assays in myotubes and adipocytes, this assay system can be used to identify potential novel therapeutic targets for the treatment of Type 2 diabetes.

**Key words:** diabetes, glucose transporter 4 (GLUT4), high-throughput screening, insulin resistance, insulin receptor substrate 1 (IRS1), therapeutic target.

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**INTRODUCTION**

GLUT4 (glucose transporter 4) plays an important role in glucose homeostasis in both animals and humans. In the basal state, GLUT4 resides in intracellular cytoplasmic vesicles. On insulin stimulation, GLUT4 translocates to the plasma membrane where it transports glucose from the extracellular environment into cells. As the only known insulin-responsive glucose transporter, GLUT4 plays a key role in insulin-mediated regulation of glucose metabolism in vivo. GLUT4 is expressed predominantly in skeletal-muscle tissues and to a lesser extent in heart and adipose tissues. Under normal physiological state, insulin-stimulated glucose uptake by GLUT4 is responsible for efficiently removing the bulk of postprandial glucose from circulation into skeletal muscle [1,2]. It was reported that mice deficient in GLUT4 develop Type 2 diabetes, whereas transgenic mice over-expressing GLUT4 have improved glucose metabolism in vivo [3–5]. Both protein levels and insulin-stimulated translocation of GLUT4 are reduced in the muscle of diabetic human subjects [2]. Due to the important role of GLUT4 in muscle action in vivo and reduced GLUT4 function in the diabetic states, therapeutics that promote insulin-stimulated GLUT4 translocation could increase postprandial glucose uptake into skeletal muscle, consequently improving insulin-sensitivity [2].

Insulin-stimulated GLUT4 translocation to the plasma membrane involves both P13K (phosphoinositide 3-kinase)-dependent and -independent pathways. Insulin stimulates autophosphorylation and activation of insulin receptors. In the P13K signalling pathway, insulin receptor phosphorhates IRS (insulin receptor substrate) proteins that bind to and activate P13K, leading to production of PIP3 (phosphatidylinositol 3,4,5-trisphosphate). PIP3 binds to and activates several downstream serine/threonine kinases including PDK1 (phosphoinositide-dependent kinase 1), Akt (also called PKB (protein kinase B)) and atypical PKCα (protein kinase C α). These serine/threonine kinases then phosphorylate and modulate downstream proteins, including those present in GLUT4 vesicles [e.g. AS160 (Akt substrate of 160 kDa)], finally leading to translocation of GLUT4 to the plasma membrane. However, there is considerable evidence suggesting that a P13K-independent pathway exists where insulin can

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**Abbreviations used:** CytoB, cytochalasin B; DMEM, Dulbecco’s modified Eagle’s medium; 2DOG, 2-deoxy-D-[1-3H]glucose; GFP, green fluorescent protein; eGFP, enhanced GFP; Eu-wMyc, DELFIA® Eu-N1 anti-c-Myc antibody; FCS, fetal calf serum; GLUT4, glucose transporter 4; HEK-293 cell, human embryonic kidney cell; HTS, high-throughput screening; IRS, insulin receptor substrate; JNK1, c-Jun N-terminal kinase 1; P13K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKCα, protein kinase C α; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTP1B, protein tyrosine phosphatase 1B; rGLUT4, rat GLUT4; siRNA, small interfering RNA; S6K1, ribosomal protein S6 kinase, 70 kDa, polypeptide 1; TRF, time-resolved fluorescence; TRITC, tetramethylrhodamine B-isothiocyanate.

† To whom correspondence should be addressed, at PO Box 2000, RY80N-A26, Merck and Co., Inc., Rahway, NJ 07065, U.S.A. (email guoqiang_jiang@merck.com).

‡ These authors have contributed equally to this work.
stimulate c-Cbl tyrosine phosphorylation, initiating a signalling cascade that involves proteins such as CAP (Cbl-associated adaptor protein), G-protein TC10 and, eventually, GLUT4 translocation in a PI3K-independent fashion [1,2,6,7].

Although progress has been made over the past 2–3 decades in uncovering the molecular mechanisms mediating insulin action and GLUT4 translocation, it remains likely that novel regulators of this pathway are undiscovered. In particular, understanding how the signalling pathway from insulin to GLUT4 is altered under diabetic states may lead to identification of potential therapeutic targets. The discovery of such novel regulators of insulin-stimulated GLUT4 translocation requires, among other things, reliable cellular assays. Historically, GLUT4 translocation assays are performed in differentiated myotubes or adipocytes [8,9]. The requirement for differentiation makes screening with such assays difficult, particularly in an HTS (high-throughput screening) mode. Differentiation extends the assay time line and makes assays more variable from well to well, plate to plate, and particularly, from batch to batch of cells.

In the present study, we stably co-expressed c-Myc and eGFP [enhanced GFP (green fluorescent protein)] dual-tagged GLUT4 and IRS1 in HEK-293 cells (human embryonic kidney cells), and showed that insulin potently stimulates both GLUT4 translocation and glucose uptake in these cells in a PI3K-dependent and Akt-dependent fashion. These cells can be rendered insulin-resistant by palmitate treatment, and the insulin-resistant cells can be effectively used to identify negative regulators of the GLUT4 translocation. Such negative regulators may serve as potential therapeutic targets for the treatment of Type 2 diabetes.

MATERIALS AND METHODS

Construction of c-Myc and eGFP dual-tagged GLUT4 expression vector

The plasmid pCX2-GLUT4myc was obtained from Dr Youseke Ebina (Division of Molecular Genetics, Institute for Enzyme Research, University of Tokushima, Tokushima, Japan). The plasmid construct encodes an rGLUT4 (rat GLUT4) cDNA with a 14-amino-acid c-Myc tag inserted in the first extracellular loop of the GLUT4 protein [10]. Using the pCX2-GLUT4myc construct as a template, PCR amplification was performed using the primers 5′-CGGAA TTC-3′ and 5′-CGGAA TTC-3′. The PCR product was then digested with EcoRI and cloned into the expression vector pEGFP-N2 (Clontech). The final construct, pEGFP-N2.rGLUT4.cMyc.eGFP, encodes an rGLUT4 with both the c-Myc tag inserted in the first extracellular loop and an eGFP tag at the C-terminus.

Generation of HEK-293 cells stably expressing recombinant GLUT4

HEK-293 cells were obtained from the A.T.C.C. HEK-293 cells stably expressing recombinant IRS1 (HEK-293.IRS1 cells) were a gift from Dr Richard Roth (Department of Medicine, Stanford University, Stanford, CA, U.S.A.) [11]. HEK-293 and HEK-293.IRS1 cells were obtained from the A.T.C.C. HEK-293 cells were obtained from the A.T.C.C. HEK-293.Glut4 cells were maintained in the same medium with the addition of 500 μg/ml G418.

Glucose uptake assay

Briefly, cells were seeded on 12-well plates at 100000 cells per well. On the next day, the cells were serum-starved for 2–3 h in DMEM plus 0.1% BSA. After washing three times with KRP buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgCl2, 1.2 mM CaCl2, 20 mM Heps and 0.1 mg/ml sodium pyruvate, pH 7.4), the cells were then incubated in KRP buffer plus vehicle, CytoB (cytochalasin B) and/or insulin for 30 min. A mixture of 2DOG (2-deoxy-D-[1-3H]glucose; Amersham) and unlabelled glucose was then added. After incubation for another 10 min, the wells were washed three times with ice-cold PBS. Lysis buffer (1% Triton X-100 plus 0.01% SDS) was then added. The lysates were cleared by centrifugation and then subjected to scintillation counting.

Cell surface binding of TRITC (tetramethylrhodamine β-isothiocyanate)-labelled c-Myc antibody: confocal imaging of fixed cells

HEK-293 cells grown on 96-well plates coated with poly-L-lysine at 100000 cells per chamber. On the next day, cells were serum-starved in DMEM plus 0.1% BSA for 3 h and stimulated with insulin for 30 min at 37°C. They were then fixed with ultrapure formaldehyde (Polysciences) for 20 min and rinsed three times in PBS and once in Detergent-free Assay Buffer. The fixed cells were then incubated with 1 μg/ml TRITC (red)-conjugated anti-c-Myc antibody (Santa Cruz Biotechnology) in the Detergent-free Assay Buffer for 1–2 h with gentle rocking, washed five times with PBS and then visualized by confocal microscopy.

Cell surface binding of EU (europium)-labelled c-Myc antibody: TRF (time-resolved fluorescence) analysis of fixed cells

HEK-293 cells grown on 96-well plates coated with poly-L-lysine were serum-starved in DMEM plus 0.1% BSA for 3 h. Cells were then stimulated with insulin for 30 min at 37°C, fixed with ultrapure formaldehyde for 20 min, rinsed three times in PBS and once in Detergent-free assay Buffer. The fixed cells were then incubated with 10 nM Eu-α-Myc (DELFIAMA Eu-N1 anti-c-Myc antibody) (PerkinElmer) in the Detergent-free assay Buffer for 1–2 h with gentle rocking, washed five times with Detergent-free Wash Buffer (PerkinElmer), and incubated with Enhance Solution (PerkinElmer) for 30 min with gentle rocking. The plates were then read with an EnVisionTM Multilabel Reader (PerkinElmer) with λex = 615 nm and λem = 340 nm.

Transfection of siRNA (small interfering RNA)

SMARTpool siRNAs from Dharmaco were used to knock down all genes of interest in the present study. A total of 20000 HEK-293 cells were seeded on to poly-L-lysine-coated 96-well plates 24 h prior to transfection. Transfection was performed using Lipofectamine™ 2000 (Invitrogen) reagents as per the manufacturer’s instructions. The final siRNA concentration in the transfection mixture was 60 nM. After a 24 h incubation with siRNAs, transfection mixture was removed, and the cells were incubated and recovered in regular growth medium for 24 h before translocation assay. Efficiency of mRNA knockdown was determined by Taqman as previously described [12] using corresponding probes purchased from Applied Biosystems.
Conjugation of palmitate to BSA and induction of insulin resistance in HEK-293 cells

The conjugation of sodium palmitate (Sigma) with BSA was performed under sterile conditions. Briefly, 200 mg of sodium palmitate was mixed with 1.25 ml of ethanol and ground until it was dissolved. Then, 1.5 g of fatty acid-free BSA powder (Sigma) and 20 ml of DMEM were added to the mixture. The mixture was thoroughly ground until it became ‘milk-shake’-like in appearance. Another 28 ml of DMEM was then added and mixed. The final solution contained 3% (w/v) BSA and 15 mM palmitate. This solution was aliquoted and stored in liquid nitrogen to prevent oxidation until use. To induce insulin resistance, cells were washed twice with DMEM plus 0.1% BSA, and then incubated with DMEM plus 0.1% BSA plus BSA-conjugated palmitate at a specified concentration and duration. The cells were then subjected to biochemical assays.

Western-blot analysis

Cell lysates were resuspended in SDS-loading buffer (Invitrogen, Carlsbad, CA, U.S.A.) and separated in precast 4–20% gradient NuPAGE SDS/PAGE gels (Invitrogen). The proteins were then transferred to PVDF membrane and probed with primary antibodies. Detection was performed with an ECF Western Blotting kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) by scanning with a Storm™ gel and blot imaging system (Molecular Dynamics) as per the manufacturer’s recommendations. Rabbit polyclonal antibodies against total IRS1 and IRS1 phosphorylated at Ser307 (IRS1-pS307) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit antibody against GFP was purchased from Santa Cruz Biotechnology.

RESULTS

HEK-293 cells stably co-expressing both IRS1 and GLUT4 (HEK-293.IRS1.GLUT4 cells) exhibit insulin-stimulated glucose uptake

An expression vector was constructed to encode rGLUT4 with a c-Myc tag inserted at the first extracellular loop and an eGFP tag at the C-terminus. The expression vector encoding the dual-tagged recombinant GLUT4 was transfected into either regular HEK-293 cells or HEK-293 cells stably expressing recombinant IRS1 (HEK-293.IRS1). Multiple rounds of FACS were performed, finally resulting in the establishment of HEK-293 cells stably expressing the dual-tagged GLUT4 alone (HEK-293.GLUT4 cells) and HEK-293 cells stably expressing both IRS1 and the dual-tagged GLUT4 (HEK-293.IRS1.GLUT4 cells). Using antiserum against GFP, Western-blot analysis on cell lysates showed that the dual-tagged GLUT4 protein is expressed to similar levels in HEK-293.GLUT4 and HEK-293.IRS1.GLUT4 cells (Figure 1A).

To determine whether the recombinant GLUT4 molecule is functional, a 2DOG uptake experiment was performed using HEK-293, HEK-293.IRS1, HEK-293.GLUT4 and HEK-293.IRS1.GLUT4 cells in the presence or absence of insulin and CytoB, a glucose uptake inhibitor. The results showed that, as expected, CytoB treatment significantly reduced basal glucose uptake in all four cell types (Figure 1B). Both HEK-293.GLUT4 and HEK-293.IRS1.GLUT4 cells showed higher basal glucose uptake than the parental HEK-293 cells and HEK-293.IRS1 cells respectively. Importantly, insulin treatment significantly increased glucose uptake only in HEK-293.IRS1.GLUT4 cells. Taken together, these results suggest that co-expression of IRS1 enables functional expression of the recombinant GLUT4 in HEK-293 cells.

Figure 1 Co-expression of IRS1 enables GLUT4 to mediate insulin-stimulated glucose uptake in HEK-293 cells

A) Western blot

B) Glucose uptake

HEK-293.IRS1.GLUT4 cells exhibit insulin-stimulated GLUT4 translocation

The above experiment demonstrated that the recombinant GLUT4 is functional in the HEK-293.IRS1.GLUT4 cells, suggesting that there was insulin-stimulated GLUT4 translocation in the cells. To directly observe and confirm such a translocation, confocal microscopy was performed on living (Figures 2A and 2B) as well as fixed HEK-293.IRS1.GLUT4 cells (Figures 2C and 2D) with or without insulin stimulation. Confocal imaging of eGFP movement was performed on the same living cells before and after insulin treatment. The results showed that, prior to insulin stimulation, vesicles with green fluorescence were seen throughout the cytoplasm of the HEK-293.IRS1.GLUT4 cells (Figure 2A). Conversely, vesicles with green fluorescence were seen mainly on or near the plasma membranes at 30 min post insulin stimulation (Figure 2B). No such vesicle translocation was observed in HEK-293.GLUT4 cells (results not shown). These results suggest that insulin stimulates translocation of GLUT4-containing vesicles to the plasma membrane.

Confocal imaging was also performed on HEK-293.IRS1.GLUT4 cells, which were treated or untreated with insulin, fixed and then stained with TRITC-labelled anti-c-Myc antibody under non-permeable detergent-free conditions. Since
Insulin stimulates GLUT4 translocation to the plasma membrane in HEK-293.IRS1.GLUT4 cells

HEK-293.IRS1.GLUT4 cells were seeded onto coverslip chambers and cultured overnight and then serum-starved for 2–3 h on the next day before insulin treatment and imaging analysis as described in the Materials and methods section. (A, B) GFP imaging. Shown are confocal images of GFP-positive GLUT4 vehicles in the same living HEK-293.IRS1.GLUT4 cell before (A) and after (B) incubation with 100 nM insulin for 30 min. (C, D) Immunohistochemical staining. Shown are confocal images of HEK-293.IRS1.GLUT4 cells untreated (basal) (C) or treated (D) with 100 nM insulin for 30 min, fixed with formaldehyde and then stained with TRITC-labelled anti-c-Myc antibody as described in the Materials and methods section. The c-Myc tag is located in the first extracellular loop of the recombinant GLUT4, only the GLUT4 proteins that are fused to the plasma membrane and have the first extracellular loop exposed to the extracellular environment will be recognized by the labelled antibody. The results showed that, indeed, insulin stimulation led to a significant increase in staining by TRITC-labelled anti-c-Myc antibody and that the staining was localized almost exclusively to the plasma membrane (compare Figure 2C with 2D). Taken together, the results from the confocal imaging on both living and fixed cells demonstrated that the dual-tagged recombinant GLUT4 proteins not only translocated to but also fused with the plasma membrane on insulin stimulation.

Development of the TRF assay for the quantification of GLUT4 translocation in HEK-293 cells

A TRF 96-well assay was developed by quantifying the binding of Eu-αMyc antibody to the c-Myc tag in recombinant GLUT4 when it is present in the extracellular environment after translocation and membrane fusion. Insulin treatment for 15 min increased cell surface binding of Eu-αMyc antibody in a concentration-dependent fashion with an EC50 of 100–300 nM, which is physiologically relevant (Figure 3A). Insulin-dependent cell surface binding of Eu-αMyc antibody was not observed in HEK-293.IRS1 cells (see Figure 5). The signal-to-noise ratios of the assay (or assay windows) were greater at higher cell densities (Figure 3A). The assay windows were largely unaffected by the presence of serum up to a concentration of 1.0% (Figure 3B).

GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is PI3K- and Akt-dependent

It is known that PI3K and Akt play important roles in insulin-stimulated GLUT4 translocation in skeletal-muscle myotubes and adipocytes both in vitro and in vivo [1]. To determine the role of PI3K in insulin-stimulated GLUT4 translocation in HEK-293.IRS1.GLUT4 cells, insulin titration was performed on cells pretreated with vehicle or the PI3K inhibitor LY294002 (Figure 4). The results showed that LY294002 pretreatment did not affect cell surface binding of Eu-αMyc antibody at basal state but significantly suppressed insulin-stimulated cell surface binding of Eu-αMyc antibody in the HEK-293.IRS1.GLUT4 cells (Figure 4A). In addition, we also tested the effect of a small-molecule Akt1 inhibitor (Akt1i-1), Akt2 inhibitor (Akt2i-2) and Akt1/2 dual inhibitor (Akt1/2i-2) [13]. The results showed that these inhibitors increasingly reduced insulin-stimulated cell surface binding of Eu-αMyc antibody (Figure 4B). Taken together, the results from the confocal imaging and TRF assay demonstrate that insulin-stimulated GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is PI3K- and Akt-dependent.

Figure 3 Quantification of GLUT4 translocation in HEK-293.IRS1.GLUT4 cells by the TRF assay

(A) HEK-293.IRS1.GLUT4 cells were seeded on to a 96-well plate at a specified cell density and cultured overnight. The cells were then serum-starved for 3 h, treated with insulin for 15 min, fixed with formaldehyde, stained with Eu-αMyc antibody and quantified using the EnVision™ Multilabel Reader as described in the Materials and methods section. (B) HEK-293.IRS1.GLUT4 cells were seeded on to a 96-well plate at the density of 80,000 cells per well and processed as in (A) except that the cells were serum-starved in medium containing 0, 0.1, 0.3 or 1.0% FCS for 3 h. MFL, mean fluorescence unit.
GLUT4 translocation high-throughput screening assay

Figure 4 GLUT4 translocation in HEK-293.IRS1 cells is PI3K- and Akt-dependent

HEK-293.IRS1.GLUT4 cells were seeded on to a poly-L-lysine-coated 96-well plate at 40,000 cells per well. On the next day, the cells were serum-starved, stimulated with insulin and processed as in Figure 3(A) except that, after serum starvation and prior to insulin treatment, cells were pretreated with DMSO, 5 μM of the PI3K inhibitor LY294002 (LY) (A) and 6 μM Akti-1 (an Akt1 inhibitor), 12 μM Akti-2 (an Akt2 inhibitor) or 2 μM Akti-1/2 (an Akt1/2 dual inhibitor) (B) for 30 min.

together, these results confirmed that insulin-stimulated GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is both PI3K- and Akt-dependent.

Palmitate treatment induces insulin resistance in HEK-293.IRS1.GLUT4 cells

Non-esterified fatty acids are known to induce insulin resistance in vitro and in vivo. Accordingly, experiments were performed to determine whether palmitate could induce insulin resistance in HEK-293.IRS1.GLUT4 cells (Figure 5). Treatments with palmitate for 6 or 20 h did not affect non-specific cell surface binding of Eu-αMyc antibody in HEK-293.IRS cells in the absence or presence of insulin. Palmitate treatments also did not affect cell surface binding of Eu-αMyc antibody to HEK-293.IRS.GLUT4 cells in the absence of insulin. Conversely, treatment with palmitate at concentrations above 6 μM reduced insulin-stimulated cell surface binding of Eu-αMyc antibody to HEK-293.IRS.GLUT4 cells in a dose-dependent fashion (Figures 5A and 5B). Additionally, Western-blot analysis showed that overnight treatment with 0.5 mM palmitate increases inhibitory phosphorylation of Ser307 in IRS1 (Figure 5C). Finally, palmitate treatment reduced insulin-stimulated Akt phosphorylation in HEK-293.IRS1.GLUT4 cells (Figure 5D). These results suggest that, as expected, palmitate treatment rendered the HEK-293.IRS1.GLUT4 cells insulin-resistant.

Knockdown of PTEN (phosphatase and tensin homologue deleted on chromosome 10) and PTB1B significantly increases GLUT4 translocation in insulin-resistant HEK-293.IRS1.GLUT4 cells

PTEN and PTP1B (protein tyrosine phosphatase 1B) have both been shown to be negative regulators of the insulin signalling pathway. Experiments were therefore performed to examine the effects of siRNA knockdown of both PTEN and PTP1B genes on basal and insulin-stimulated GLUT4 translocation in HEK-293.IRS1.GLUT4 cells (Figure 6). These siRNAs reduced the mRNA levels of PTEN and PTP1B by 93 and 82 % respectively. Insulin stimulation resulted in ~4-fold increases in the cell surface binding of Eu-αMyc antibody in HEK-293.IRS1.GLUT4 cells without palmitate treatment, but only ~2-fold increases in cells with palmitate treatment. PTEN and PTP1B siRNA did not significantly affect insulin-stimulated cell surface binding of Eu-αMyc antibody in HEK-293.IRS1.GLUT4 cells without palmitate treatment. In contrast, both siRNAs significantly increased insulin-stimulated cell surface binding of Eu-αMyc antibody in HEK-293.IRS1.GLUT4 cells treated with palmitate. These results provide the first evidence that HEK-293.IRS1.GLUT4 cells pretreated with palmitate can serve as an improved HTS-compatible cellular system to identify negative regulators of the GLUT4 translocation pathway.

Knockdown of JNK1 (c-Jun N-terminal kinase 1), S6K1 (ribosomal protein S6 kinase, 70 kDa, polypeptide 1) and PKC θ by siRNA increases GLUT4 translocation in insulin-resistant HEK-293.IRS1.GLUT4 cells

To validate the feasibility of using insulin-resistant HEK-293.IRS1.GLUT4 cells with palmitate pretreatment to find negative regulators of insulin-stimulated GLUT4 translocation, the effects of siRNA knockdown of JNK1, S6K1 and PKC θ were also examined (Figure 7). JNK1, S6K1 and PKC θ are serine kinases that have been reported to mediate fatty acid-induced cellular insulin resistance [14–22]. These siRNAs reduced the mRNA levels of JNK1, S6K1 and PKC θ by 74, 77 and 73 % respectively. It was expected that knockdown of these molecules would reduce palmitate-induced insulin resistance and improve insulin-stimulated GLUT4 translocation in the HEK-293.IRS1.GLUT4 cells. The results showed that JNK1, S6K1 and PKC θ siRNAs reduced the expression levels of their target genes by ~75 %, and significantly increased insulin-stimulated cell surface binding of Eu-αMyc antibody on cells treated with palmitate.

DISCUSSION

In the present study, both a c-Myc and eGFP dual-tagged recombinant GLUT4 and recombinant IRS1 were stably co-expressed
in HEK-293 cells (HEK-293.IRS1.GLUT4 cells). Several lines of evidence demonstrate that the recombinant GLUT4 is functional in these cells. First, insulin significantly increased glucose uptake in the HEK-293.IRS1.GLUT4 but not in HEK-293.GLUT4 cells (Figure 1). Secondly, confocal imaging of living HEK-293.IRS1.GLUT4 cells demonstrated an insulin-stimulated translocation of eGFP-positive vesicles from the cytoplasm to the plasma membrane (Figures 2A and 2B). Thirdly, confocal imaging of fixed HEK-293.IRS1.GLUT4 cells showed an insulin-stimulated increase in cell surface binding by anti-c-Myc antibody (Figures 2C and 2D). These findings support the proposition that, in response to insulin stimulation, the recombinant GLUT4 in the HEK-293.IRS1.GLUT4 cells translocates from cytoplasmic vesicles to the plasma membrane, fuses with the plasma membrane and therefore exposes the c-Myc tag to the extracellular environment while functionally transporting glucose into the cells.

Traditionally, GLUT4 translocation assays are performed in differentiated adipocytes or myotubes. Such cells are relevant to GLUT4 function in vivo, since the transporter is selectively expressed in adipose and muscle tissues in human and non-human species. In contrast, GLUT4 is not significantly expressed in HEK-293 cells and has not been reported to play a role in glucose metabolism in kidney, from which HEK-293 cells are derived. Therefore HEK-293.IRS1.GLUT4 cells may be seen as a somewhat artificial system in which to study GLUT4 translocation. However, our results indicate that GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is mediated by a molecular signalling cascade similar to that in adipocytes and myotubes. First, GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is induced by insulin, the key stimulator of GLUT4 translocation in vivo (Figures 1 and 2). Secondly, GLUT4 translocation in HEK-293.IRS1.GLUT4 cells is inhibited by both a PI3K inhibitor and multiple Akt inhibitors (Figure 4), but increased by siRNAs of both PTEN and PTP1B (Figure 6). Therefore it appears that recombinant GLUT4 functionally translocates in the HEK-293.IRS1.GLUT4 cell lines via similar molecular signalling mechanisms that govern GLUT4 translocation in adipose and muscle cells and tissues in vitro and in vivo. Nevertheless, it is expected that HEK-293.IRS1.GLUT4 cells may lack certain components of the insulin signalling pathway present in either adipocytes or myocytes where GLUT4 is naturally expressed and functional.

The TRF-based GLUT4 translocation assay in HEK-293.IRS1.GLUT4 cells is easily executed in a 96-well high-throughput format. The assay eliminates the need for lengthy differentiation that is required for experiments utilizing adipocytes and myotubes, resulting in a significant reduction in overall assay time as well as assay variability. Indeed, data generated from our TRF assay is highly reproducible from well to well, plate
Figure 6 Knockdown of PTEN and PTP1B by siRNA increases insulin-stimulated GLUT4 translocation in HEK-293.IRS1.GLUT4 cells treated with palmitate

HEK-293.IRS1.GLUT4 cells were seeded on to poly-L-lysine-coated 96-well plates at 20,000 cells per well on day 1. On day 2, the cells were transfected with SMARTpool non-targeting control siRNA (NT), PTEN siRNA or PTP1B siRNA overnight using Lipofectamine™ 2000. On day 3, the transfected medium was replaced with regular growth medium. On day 4, the cells were switched to serum-free medium without or with 0.5 mM BSA-conjugated palmitate. On day 5, cells were stimulated with 10 nM insulin and processed as in Figure 3(A). Also shown are percentages of reduction of PTEN and PTP1B expression as determined by Taqman analysis. The percentages of inhibitions were calculated relative to non-targeting siRNA-transfected cells.

Importantly, transfection with siRNA of both PTEN and PTP1B, known inhibitors of the insulin signalling pathway, significantly increased GLUT4 translocation in cells treated with palmitate but not in untreated cells (Figure 6). Additionally, similar results were observed on treatment with siRNA of JNK1, S6K and PKCθ (Figure 7). We therefore concluded that, by rendering HEK-293.IRS1.GLUT4 cells insulin-resistant by palmitate treatment, this translocation assay can be readily used to discover negative regulators of the insulin signalling and the GLUT4 translocation pathway.

In summary, we have developed a GLUT4 translocation system in HEK-293 cells co-expressing recombinant GLUT4 and IRS1. Insulin stimulates GLUT4 to translocate to the membrane and functionally transport glucose in a PI3K- and Akt-dependent fashion. In comparison with assays using differentiated adipocytes or myotubes, the current assay is less resource-intensive, more reproducible and more compatible with the HTS format. This novel HTS assay system can be used to effectively identify novel negative regulators of the insulin signalling and the GLUT4 translocation pathway when it is performed on HEK-293.IRS1.GLUT4 cells made insulin-resistant by palmitate treatment. Hits identified in this assay can be further validated in more physiologically relevant adipose and muscle cell lines, and if confirmed, may represent potential novel targets for the treatment of Type 2 diabetes in humans.
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