

GLUT4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay

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Abstract We report a rapid and sensitive colorimetric approach to quantitate the amount of glucose transporters exposed at the surface of intact cells, using L6 muscle cells expressing GLUT4 containing an exofacial myc epitope. Unstimulated cells exposed to the surface 5 fmol GLUT4myc per mg protein. This value increased to 10 fmol/mg protein in response to insulin as 2-deoxyglucose (10 μ M) uptake doubled. The results are substantiated by immunofluorescent detection of GLUT4myc in unpermeabilized cells and by subcellular fractionation. We further show that wortmannin and the cytoskeleton disruptors cytochalasin D and latrunculin B completely blocked these insulin effects. The rapid quantitative assay described here could be of high value to study insulin signals and to screen for potential anti-diabetic drugs.

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Key words: Glucose transport; Cell surface; Plasma membrane; Actin cytoskeleton; Latrunculin B

1. Introduction

Insulin rapidly stimulates the rate of glucose uptake into the major glucose consuming tissues, fat and muscle. The insulin-stimulated glucose uptake mainly results from the translocation of GLUT4 from an intracellular storage pool to the plasma membrane. Several subcellular fractionation studies have demonstrated an increase in GLUT4 in a plasma membrane-enriched fraction in response to insulin, which cannot be accounted for by the new synthesis of glucose transporter proteins [1–3], but rather by the redistribution of GLUT4 from an internal pool to the plasma membrane [4–7]. In type 2 diabetes mellitus, fat cells and muscle fibers appear to be insensitive to the hormone [8–10], a defect that has been associated with impaired GLUT4 translocation [11,12]. To date, the exact molecular mechanism by which insulin induces the translocation of GLUT4 to the plasma membrane is not fully understood, although it is known that stimulation of glucose uptake and GLUT4 translocation require the activation of phosphatidylinositol 3-kinase [13,14] and the existence of an intact actin network [15]. An understanding of the molecular mechanisms of insulin-stimulated glucose transport might provide insights into the insensitivity of target cells in type 2 diabetes.

The L6 muscle cell line has been used to study skeletal muscle glucose metabolism in response to acute insulin stimulation [1,2,16]. Subcellular fractionation followed by immunoblotting as well as photoaffinity labeling followed by immunoprecipitation has demonstrated that the hormone causes

rapid translocation of GLUT4 to the plasma membrane [16–18]. These manipulations are lengthy and could introduce factors which may lead to inaccuracy. An ideal method should allow one to immunologically detect GLUT4 on the surface with an anti-exofacial antibody specific for GLUT4.

We report the development of a simple method for the rapid detection of GLUT4 translocation in cells in culture of high sensitivity, which allows for the accurate calculation of the number of molecules of GLUT4 at the cell surface. We use this method to demonstrate a requirement for an intact cytoskeletal network in the translocation of GLUT4.

2. Materials and methods

2.1. Materials

o-Phenylenediamine dihydrochloride (OPD) and peroxidase-coupled donkey anti-mouse IgG was obtained from Sigma (St. Louis, MO). C-myc peptide and monoclonal antibody 9E10 were purchased from Genosys (Woodland, TX), and Santa Cruz (Santa Cruz, CA) respectively. Cy3-conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA). Antifade kit was purchased from Molecular Probes (Leiden, The Netherlands).

2.2. Construction of L6 cells expressing c-myc epitope-tagged GLUT4 (GLUT4myc)

GLUT4myc cDNA was constructed by inserting the human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4, as described [19]. The epitope does not affect GLUT4 activity [19]. GLUT4myc cDNA was subcloned into the mammalian expression vector pCXN (pCXN-GLUT4myc) [20]. L6 myoblasts were transfected with pCXN-GLUT4myc and pSV2-*bsr*, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride (Funakoshi, Tokyo).

2.3. Cell culture

L6-GLUT4myc myoblasts in cell monolayers were maintained in α -MEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO₂ and 90% air at 37°C. Cells were grown in 15 cm² dishes for subcellular fractionation experiments, in 12 or 24 well plates for glucose uptake and immunocolorimetric assays, and on glass coverslips for immunocytochemistry. Prior to experiments, cells were incubated with serum-free α -MEM supplemented with 25 mM glucose for 5 h.

2.4. 2-Deoxy-[³H]D-glucose uptake

Hexose uptake was measured at room temperature for 5 min in transport buffer containing: 20 mM HEPES, pH 7.4 and 10 μ M 2-deoxy-[³H]D-glucose (1 mCi/ml) as described previously [2]. Cellular protein content was measured by the bicinchoninic acid method [21].

2.5. Indirect immunofluorescence

Quiescent L6-GLUT4myc cells grown on glass coverslips were treated as described in each experiment, then rinsed once with PBS, fixed with 3% paraformaldehyde in PBS for 3 min at room temperature, and neutralized with 1% glycine in PBS at 4°C for 10 min. The cells were incubated with PBS containing 10% goat serum and 3% BSA at 4°C for at least 30 min. Primary antibody (anti-c-myc 9E10) was added at a dilution of 1:100 and maintained at 4°C for 30 min. The cells were extensively washed with cold PBS before introducing

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the secondary antibody (Cy3-IgG, 1:1000) for 30 min at 4°C. The cells were washed, then mounted and immediately examined by immunofluorescence microscopy.

2.6. Subcellular fractionation and immunoblotting

Membrane preparations were performed as described previously for wild type L6 cells [22]. Equal amounts of membrane proteins were analyzed by SDS-PAGE and electrotransferred onto PVDF filters as described [22]. Immunoreactive bands were visualized using either HRP-conjugated sheep anti-mouse IgG for monoclonal antibodies or HRP-conjugated goat anti-rabbit IgG for polyclonal antibodies, with the enhanced chemiluminescence (ECL) detection technique. Developed films were scanned and quantitated using NIH software.

2.7. Colorimetric assay of surface GLUT4myc

Quiescent L6-GLUT4myc cells treated as indicated were washed once with PBS, fixed in 3% paraformaldehyde in PBS for 3 min at room temperature, and the fixative was immediately neutralized by incubation with 1% glycine in PBS at 4°C for 10 min. The cells were blocked with 10% goat serum and 3% BSA in PBS at 4°C for at least 30 min. Primary antibody (anti-c-myc, 9E10) was then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4°C. The cells were extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1000). After 30 min at 4°C, the cells were extensively washed and 1 ml OPD reagent was added to each well. The colorimetric reaction was stopped by addition of 0.25 ml of 3 N HCl for 10 min at room temperature. The supernatant was collected and the optical absorbance was measured at 492 nm. Standard curves were generated using either peroxidase conjugated anti-mouse IgG alone or myc-tag peptide, as indicated. Myc-tag peptide at various concentrations was coated onto 24-well plates as indicated by incubation at 4°C for 24 h then allowed to dry. The plates were rinsed with PBS to move excess salt and the uncoated spaces were blocked with 10% goat serum and 3% BSA.

2.8. Statistical analysis

Student's *t*-test was used using Excel software.

3. Results

3.1. Hexose transport

Under basal conditions, L6-GLUT4myc myoblasts took up glucose at a rate of 11.7 ± 0.6 pmol/min/mg protein. Insulin significantly increased glucose uptake in a time-dependent manner which peaked at 30 min (data not shown). At this time, insulin stimulated glucose uptake to 22.6 ± 0.8 pmol/min mg protein, representing a 193% rise over basal values ($P < 0.05$) (Fig. 1a). The increase in glucose uptake was significantly decreased upon pre-treatment with 1 μ M cytochalasin D ($P < 0.05$, Fig. 1a), a drug which has been previously reported to inhibit specifically the increase in glucose transport in L6 myotubes by disruption of actin filaments [15].

Furthermore, pre-treatment of cells with latrunculin B, a structurally unrelated drug which inhibits polymerization of actin filaments [23], resulted in a similar decrement of insulin-stimulated glucose uptake ($P < 0.05$, Fig. 1a). The stimulation by insulin was also significantly decreased ($P < 0.05$, Fig. 1a) upon pre-treatment of the cells with wortmannin, a drug which is known to block glucose uptake in muscle and fat cells by inactivating phosphatidylinositol 3-kinase [13,14].

3.2. Subcellular fractionation

Immunoblot analysis of equal amounts of proteins of the LDM and PM subcellular fractions showed a gain in GLUT4myc in isolated plasma membrane fractions from insulin stimulated cells, and a concomitant decrease in the low density microsome fractions. Densitometric analysis of these blots showed that the gain in GLUT4myc in the plasma membrane and the loss in the internal membranes were statistically significant ($P < 0.05$) (Fig. 1b,c). Pre-treatment with cytochalasin D or wortmannin significantly inhibited the insulin-mediated increase of GLUT4myc in the plasma membrane fraction, and prevented the insulin-dependent loss of GLUT4myc from the light density microsomes (Fig. 1b, $P < 0.05$). Latrunculin B behaved similarly to cytochalasin D in these subcellular fractionation experiments (Fig. 1c, $P < 0.05$).

In one experiment, the immunoblots were also probed with an anti-GLUT4 antibody. Two bands of comparable intensities were detected: the endogenous GLUT4 and the GLUT4myc which had a slower migration on SDS-PAGE. The endogenous GLUT4 had relative values of 1.0, 0.6, 0.9, and 1.1 in the LDM and 0.7, 0.9, 0.7 and 0.6 in the PM from basal, insulin, cytochalasin D-insulin and wortmannin-insulin treated cells. These values compare very closely with the relative values of GLUT4myc shown in Fig. 1b (1.0, 0.6, 1.1, 1.1, and 0.7, 1.0, 0.7 and 0.6). Therefore, the transfected GLUT4myc behaves similarly to the endogenous transporter.

3.3. Immunostaining of intact cells

Non-permeabilized cells were reacted with anti-myc antibody (9E10) which was subsequently detected with Cy3-conjugated goat anti-mouse IgG as described in Section 2. In the basal state, a low level of surface GLUT4 was detected (Fig. 2b), and the intensity of the staining increased upon insulin stimulation (Fig. 2c). In contrast, insulin did not affect the background surface staining in wild type L6 cells (Fig. 2a). Moreover, using this detection system it was demonstrated that insulin did not increase the amount of GLUT4myc at

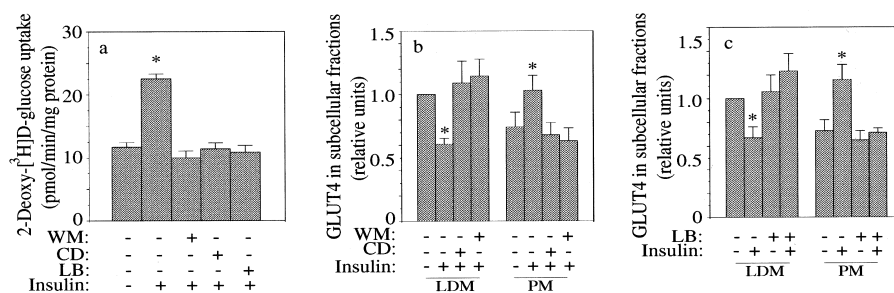


Fig. 1. 2-Deoxy-[³H]D-glucose uptake in L6-GLUT4myc cells. Cells were serum-deprived for 5 h. In the last 2 h cells were treated with or without cytochalasin D or latrunculin B, or for 20 min with or without wortmannin at the end of the 5 h period. The cells were then treated with or without insulin (100 nM) for 30 min at 37°C. a: Glucose uptake was measured at room temperature for 5 min in transport buffer as described in Section 2. b, c: Detection of GLUT4 by immunoblotting of subcellular fractions of GLUT4myc cells.

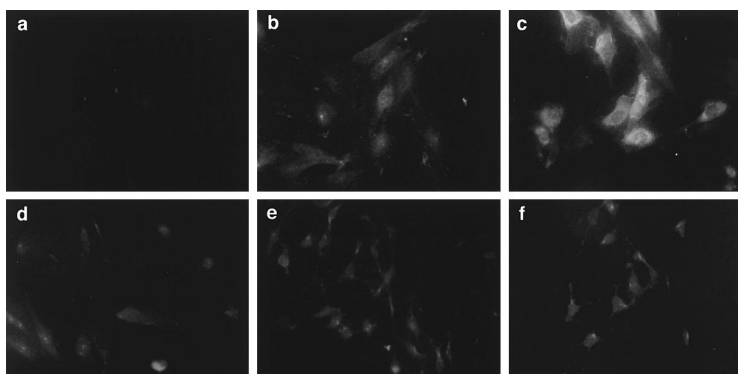


Fig. 2. Immunostaining of GLUT4 in non-permeabilized L6-GLUT4myc cells. Intact L6-GLUT4myc cells were stained with anti-myc mouse IgG (9E10), and subsequently with Cy3 goat anti-mouse IgG to detect surface GLUT4 as described in Section 2. a: Wild type L6 cells. b: Untreated L6-GLUT4myc cells. c: L6-GLUT4myc cells stimulated with insulin. d: Wortmannin pre-treated L6-GLUT4myc cells stimulated with insulin. e: Cytochalasin D pre-treated L6-GLUT4myc cells stimulated with insulin. f: Latrunculin B pre-treated L6-GLUT4myc cells stimulated with insulin. All insulin (100 nM) treatments were for 30 min at 37°C. The results shown are representative of five independent experiments.

cell surface when L6-GLUT4myc cells were pre-treated with wortmannin (Fig. 2d), cytochalasin D (Fig. 2e) or latrunculin B (Fig. 2f).

3.4. Colorimetric quantitation of surface GLUT4myc

L6-GLUT4myc myoblasts were stimulated with insulin for 30 min at 37°C. Anti-myc antibody 9E10 was added at various dilutions and allowed to interact with the cells for 30 min at 4°C, followed by anti-mouse IgG conjugated to peroxidase and then exposed to OPD as described in Section 2. The reaction product increased linearly with the concentration of the primary antibody to reach a maximum at a dilution of 1:100 (Fig. 3a). This dilution of 9E10 antibody was used for all subsequent experiments. Fig. 3b,c shows the calibration curves for the immunocolorimetric assay. In Fig. 3b, increasing concentrations of myc peptide were coated onto 24 well plates and any uncoated space was blocked with blocking buffer as described in Section 2. The plates were then treated with primary and secondary antibodies as described above for intact cells. The optical absorbance was linear for up to 3 fmol. In Fig. 3c, myc protein was omitted but increasing concentrations of peroxidase-coupled anti-mouse IgG were tested. Optical absorbance was linear for up to 6 fmol IgG.

Table 1 quantitates the amount of GLUT4myc at the surface of intact cells in response to insulin, as well as the effects of pre-treatments with wortmannin, cytochalasin D or latrunculin B, using the colorimetric method described above. Under basal conditions, the optical density per well was 0.27 ± 0.04 ; insulin stimulation caused a significant increase ($P < 0.01$) in optical absorbance to 0.53 ± 0.03 . This increase was fully blocked by pre-treatment with wortmannin (0.22 ± 0.03), cytochalasin D (0.25 ± 0.03) or latrunculin B (0.25 ± 0.03). To quantitate the amount of GLUT4myc trans-

located to the plasma membrane, the spectrophotometric readings were referred to the calibration curves from Fig. 3b,c. Both standard curves yielded similar results, indicating that the anti-myc antibody reacts with a close to 1:1 stoichiometry to its antigen. In the basal state, the amount of cell surface GLUT4myc was calculated to be about 5 fmol/mg cellular protein. This value was approximately doubled by the insulin treatment. Pre-treatment with wortmannin, cytochalasin D or latrunculin B prevented the insulin effect.

Finally, to calculate the percentage that the surface GLUT4myc represents of the total cellular GLUT4myc, the colorimetric assay of GLUT4myc was performed in intact cells and in cells permeabilized with 0.1% Triton X-100. The ratio of optical absorbance of intact:permeabilized cells was 1.0:2.6 in the basal state.

4. Discussion

Understanding the molecular mechanisms of insulin action has been of major interest since the discovery, several decades ago, that insulin stimulates glucose transport *in vivo*. Subcellular fractionation, cell photolabeling coupled to immunoprecipitation and immunofluorescence or immunoelectron microscopy have been used to detect translocation of the GLUT4 glucose transporter to the cell surface [17,18,22,24]. All of these methods are laborious and suffer from methodological inaccuracies. Subcellular fractionation is cumbersome and produces membranes that are rarely pure; moreover, quantitative recovery of all membrane compartments is difficult or impossible. Affinity labeling of surface glucose transporters followed by immunoprecipitation depends on the ability to obtain quantitative immunoprecipitation and recovery upon SDS-PAGE. This technique results in the incorporation

Table 1
Colorimetric detection of GLUT4myc on the surface of L6-GLUT4myc cells

| | Basal | Insulin | WM/Insulin | CD/Insulin | LB/Insulin |
|--|-----------------|------------------|-----------------|-----------------|-----------------|
| Optical absorbance (mean \pm S.E.M.) | 0.27 ± 0.04 | 0.52 ± 0.02 | 0.22 ± 0.03 | 0.25 ± 0.03 | 0.25 ± 0.03 |
| Relative protein change (fmol/mg protein) calibrated using Fig. 3c | 5.51 ± 0.82 | 10.82 ± 0.41 | 4.92 ± 0.62 | 5.45 ± 0.62 | 5.21 ± 0.63 |
| Relative protein change (fmol/mg protein) calibrated using Fig. 3b | 4.32 ± 0.66 | 9.51 ± 0.36 | 3.50 ± 0.46 | 4.32 ± 0.51 | 4.05 ± 0.49 |

L6-GLUT4myc cells pre-treated with or without wortmannin (WM), cytochalasin D (CD) or latrunculin B (LB) were incubated with 100 nM insulin at 37°C for 30 min. The cells were fixed and incubated with anti-myc antibody (9E10) as described in Section 2. Optical absorbance values represent the mean \pm S.E.M. of four separate experiments. The spectrophotometric readings were referred to the calibration curves from Fig. 3.

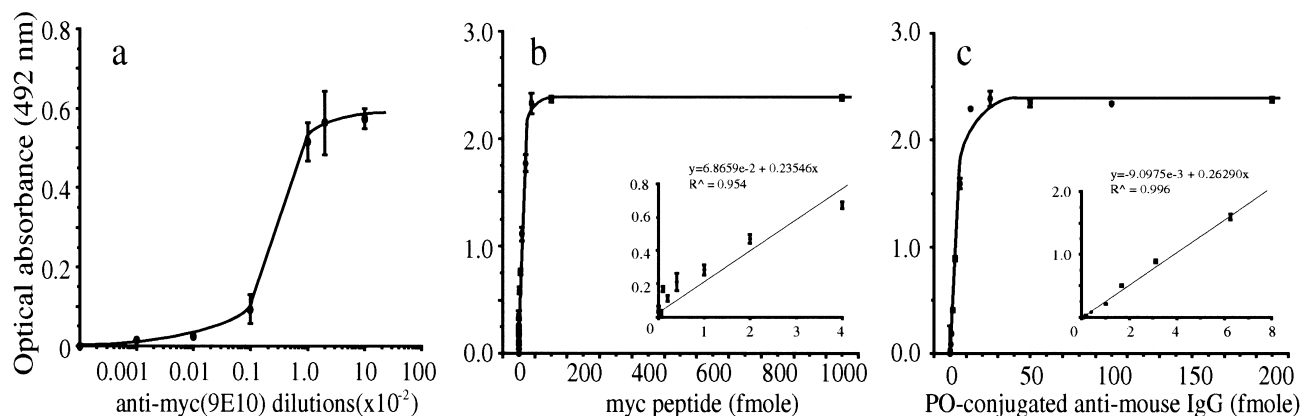


Fig. 3. Calibration of the reaction of anti-myc antibody (9E10) with myc epitope. a: L6GLUT4myc myoblasts incubated with 100 nM insulin at 37°C for 30 min were labelled with anti-myc antibody (9E10), and peroxidase-conjugated secondary sheep-anti mouse IgG as described in Section 2. The reaction of OPD was measured at 492 nm. Results are the mean \pm S.E.M., $n=3$. b: In cell-free wells, c-myc peptide at various concentrations was coated onto 24 well plates, blocked and reacted with anti-myc 9E10 antibody and peroxidase-conjugated secondary antibody followed by OPD as described above. c: Direct incubation of various concentrations of peroxidase-conjugated sheep anti-mouse IgG with OPD colorimetric reagent.

of about 1/10 000 of the label added, thus the signal to noise ratio is low; moreover, the reactivity of the photolabel can depend on the level of activity of the transporter [25] in addition to the amount of transporter exposed at the cell surface. Immunofluorescence detection does not distinguish the native GLUT4 molecules incorporated into the cell membrane from molecules in subplasmalemmal vesicles, and is not a quantitative technique. Immunogold electron microscopy detects antigens at the plasma membrane accurately, but has not been successfully used in a quantitative fashion. None of the above techniques is suitable for large numbers of experiments, as would be required for screening of anti-diabetic drugs or of agents interfering with insulin signaling or intracellular traffic. Therefore, the aim of the present study was to develop a fast and quantitative approach to measure GLUT4 translocation in intact cells. The method described here uses cells in culture and does not require subsequent immunoprecipitation, SDS-PAGE or use of radioactivity, as required by the photolabeling technique. It does not require large amounts of cells nor laborious subcellular fractionation. It is rapid, sensitive and quantitative.

Introduction of a tag c-myc epitope (14 amino acids) into the first exofacial loop of GLUT4 cDNA allows for the direct detection of functional GLUT4 translocation to the plasma membrane in intact cells. The efficacy of the method was compared with that of the conventionally used subcellular fractionation and immunofluorescence approaches. Virtually similar effects of insulin were detected by all three approaches, and all three approaches showed that the insulin effect was obliterated by pre-treatment of the cells with wortmannin or the cytoskeletal disassembling drug cytochalasin D. We have recently reported that cytochalasin D prevents arrival of phosphatidylinositol 3-kinase to the GLUT4-containing vesicles of 3T3-L1 adipocytes [26]. Here we show that latrunculin B, a drug that leads to disassembly of the cytoskeleton by a mechanism different from that of cytochalasin D, also prevents GLUT4 translocation.

Previous studies have shown that in CHO cells or other fibroblasts stably expressing heterologous GLUT4 [27,28], insulin-stimulated translocation of the transfected GLUT4 was not detectable by immunoblot analysis of subcellular frac-

tions, nor by immunofluorescence microscopy [28]. However, transfection of GLUT4myc allowed for detection of the exofacial epitope by ^{125}I -labeled secondary antibody. By this approach, insulin-induced GLUT4myc translocation was observed in CHO cells [19]. In the present study, GLUT4myc was introduced into L6 insulin-sensitive muscle cells which express endogenous GLUT4 as well as GLUT1 and GLUT3. The amount of GLUT4myc expressed at the surface of basal cells (5 fmol/mg protein) was in the range of that of native GLUT4 (about 12 fmol/mg, see [16]). This discrete level of expression of GLUT4myc likely allows for its correct localization without saturation of the proteins determining its intracellular sorting. The amount of hexose taken up during the 2-deoxyglucose uptake assay is a reflection of uptake through all transporters expressed in the cell.

GLUT4 translocation is a complex vesicular traffic process which includes fusion and docking steps. Herbst et al. [29] showed previously that introduction of a peptide corresponding to tyrosine-phosphorylated IRS-1 motifs into 3T3-L1 adipocytes resulted in a significant increase in the translocation of GLUT4 to the plasma membrane by immunofluorescence staining which mimicked the response to insulin. However, no relative increase in the rate of glucose uptake was observed in those cells. This led to the speculation that some stimulus may bring GLUT4 vesicles to the plasma membrane (docking) but is not sufficient to trigger fusion with the membrane. A converse phenomenon was observed by others upon introducing isoproterenol [30] or isoprenaline [25] to rat adipocytes whereby the insulin-dependent gain in glucose transporter detected in isolated plasma membrane was not changed while glucose transport was decreased. It is therefore conceivable that immunostaining of isolated membranes or of permeabilized cells cannot distinguish GLUT4 proteins docked on the plasma membrane from those which are fully fused with it. The detection of cell surface GLUT4 transporters achieved by the approach described in this report detects GLUT4 fully incorporated into the plasma membrane.

In summary, we have developed a colorimetric assay for the direct detection of GLUT4 translocation to the cell plasma membrane in L6 muscle cells stably expressing GLUT4myc. The results suggest that these transfected L6 cells possess the

basic machinery required for translocation of GLUT4myc and, furthermore, the L6-GLUT4myc cell system appears to be a sensitive and reliable in vitro model for studying the molecular mechanisms of insulin action on GLUT4 translocation, and for testing agents which may improve or impair insulin action.

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