Insulin Regulates the Membrane Arrival, Fusion, and C-terminal Unmasking of Glucose Transporter-4 via Distinct Phosphoinositides*

Received for publication, January 14, 2005, and in revised form, June 6, 2005 Published, JBC Papers in Press, June 13, 2005, DOI 10.1074/jbc.M500501200

Manabu Ishiki‡‡§, Varinder K. Randhawa‡‡§, Vincent Poon‡‡, Lellean JeBailey‡‡§‡‡, and Amira Klip‡‡§‡‡

From the ‡Programme in Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8 and the §Department of Biochemistry, the University of Toronto, Toronto, Ontario M5S 1A8, Canada

Insulin increases glucose uptake into muscle via glucose transporter-4 (GLUT4) translocation to the cell membrane, but the regulated events in GLUT4 traffic are unknown. Here we focus on the role of class IA phosphatidylinositol (PI) 3-kinase and specific phosphoinositides in the steps of GLUT4 arrival and fusion with the membrane, using L6 muscle cells expressing GLUT4myc. To this end, we detected the availability of the myc epitope at the cell surface or intravesicular spaces and of the cytosol-facing C-terminal epitope, in cells and membrane lawns derived from them. We observed the following: (a) Wortmannin and LY294002 at concentrations that inhibit class IA PI 3-kinase reduced but did not abate the C terminus gain, yet the myc epitope was unavailable for detection unless lawns or cells were permeabilized, suggesting the presence of GLUT4myc in docked, unfused vesicles. Accordingly, GLUT4myc-containing vesicles were detected by immunoelectron microscopy of membranes from cells pretreated with wortmannin and insulin, but not insulin or wortmannin alone. (b) Insulin caused greater immunological availability of the C terminus than myc epitopes, suggesting that C terminus unmasking had occurred. Delivering phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) to intact cells significantly increased lawn-associated myc signal without C terminus gain. Conversely, phosphatidylinositol 3-phosphate (PI3P) increased the detection of C terminus epitope without any myc gain. We propose that insulin regulates GLUT4 membrane arrival, fusion, and C terminus unmasking, through distinct phosphoinositides. PI(3,4,5)P3 causes arrival and fusion without unmasking, whereas PI3P causes arrival and unmasking without fusion.

Insulin promotes the uptake of glucose into muscle and fat tissues through a rapid gain in surface-bound glucose transporters (1–3). The muscle- and fat-specific glucose transporter GLUT4 cycles continuously between the plasma membrane and intracellular stores, with the steady-state distribution largely favoring the latter. Insulin changes this steady-state resulting in a net gain in surface GLUT4 (4–6) largely as a result of enhancing the exocytic rate of GLUT4 cycling (7, 8). Of significance, insulin resistance and diabetes are accompanied by defective GLUT4 gain at the plasma membrane of muscle and fat cells (9–11).

It is well established that signaling from class IA phosphatidylinositol (PI) 3-kinase is required for the insulin-dependent net gain in surface GLUT4 (12–15), but the specific steps in GLUT4 cycling that are regulated are not elucidated. We have recently shown that class IA PI 3-kinase is required for the insulin-dependent acceleration of GLUT4 transit through the recycling endosome (16). A second input of class IA PI 3-kinase in muscle cells is the spatial-temporal actin remodeling and its possible contribution to segregating specific signaling molecules (17, 18). However, it is not known whether fusion of insulin-sensitive GLUT4 vesicles with the plasma membrane is a regulated step, nor which phosphoinositides participate in this event. Indeed, class II PI 3-kinase C2a is also activated by insulin (19). The major product of class IA PI 3-kinase in vivo is PI(3,4,5)P3 (20, 21) and that of class II PI 3-kinase C2a (19) is PI3P, whereas both enzymes can lead to the formation of PI 3,4-bisphosphate.

Here we implement the simultaneous detection of exofacially and cytosolically facing epitopes of GLUT4myc to score the arrival and fusion of GLUT4myc-containing vesicles at the plasma membrane of myotubes, and the effect of insulin, PI(3,4,5)P3, and PI3P, in these events. An myc epitope in the first exofacial loop of GLUT4myc would face the extracellular milieu in intact cells and the intravesicular space in adhered/docked vesicles, whereas the C terminus of the transporter would always face the cytosol. Epitopes were immunodetected in membrane lawns and in intact or permeabilized muscle cells. We show that wortmannin (100 nM) and LY294002 (25 μM) cause accumulation below the plasma membrane of unfused GLUT4-containing vesicles. These results suggest that class IA PI 3-kinase regulates the membrane fusion step, because class II PI 3-kinase-C2a is not affected by these doses of the inhibitors (22). Strikingly, the C terminus of GLUT4myc was more available for antibody recognition after insulin action, suggesting unmasking of this epitope in response to the hormone.

* This work was supported by the Canadian Institutes of Health Research (CIHR) (Grant MT7307 to A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This report is dedicated to the memory of Dr. Tetsuro Kono.

§ Supported by a fellowship from the Canadian Diabetes Association.
‡‡ Supported by a CIHR Doctoral Award Studentship.
** Supported by a studentship from the Ontario Student Opportunity Trust Fund-Hospital for Sick Children Foundation Student Scholarship Program.
‡‡ To whom correspondence should be addressed: Programme in Cell Biology, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Tel.: 416-813-6392; Fax: 416-813-5028; E-mail: amira@sickkids.ca.

1 The abbreviations used are: GLUT4, glucose transporter-4; PI, phosphatidylinositol; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate, PI3P; phosphatidylinositol 3-phosphate, PBS; phosphate-buffered saline, PFA, para-formaldehyde; ANOVA, analysis of variance; GFP, green fluorescent protein; eGFP, enhanced GFP.

28792 This paper is available on line at http://www.jbc.org
Carrier delivery of PI(3,4,5)P3 caused significant GLUT4myc arrival and membrane fusion while barely allowing C-terminal detection. Conversely, PI3P caused GLUT4myc arrival and C-terminal exposure but not fusion. We propose that insulin regulates GLUT4 membrane fusion, fusion, and C-terminal unmasking, through distinct phosphoinositides.

**EXPERIMENTAL PROCEDURES**

**Reagents and Constructs—**PI(3,4,5)P3, PI(3,4)P2, PI(4,5)P2, and carrier (neomycin) were from Echelon Biosciences Inc. (Salt Lake City, UT). Monoclonal (9E10) and polyclonal (A-14) anti-myc antibodies, polyclonal (H-61) antibody against the intracellular loop region (amino acids 236–290) of GLUT4, and polyclonal (N-20) antibody sc1606 against the first exofacial loop region of GLUT4 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-GLUT4 anti-serum was raised to the 12 C-terminal residues of GLUT4 (23). Polyclonal antibody against caveolin was from Upstate Biotechnology (Lake Placid, NY). Enhanced green fluorescent protein fused to mycGLUT4 (GLUT4myc-eGFP), a kind gift from Dr. J. Pessin (SUNY, Stonybrook, NY), was subcloned into pcDNA3 (24).

**Cell Culture, cDNA Transfections, and Phosphoinositide Delivery—**L6 rat myoblasts stably expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc) and L6-wild-type myoblasts were differentially labeled from eGFP-GLUT4myc-eGFP cDNA using Lipofectamine 2000 (Invitrogen) as specified by the manufacturer, and analyzed 48 h post-transfection. Distinct phosphatidylinositol phosphates and carrier (neomycin) were prepared and applied to cells essentially as described previously (27). Neomycin was used as carrier to deliver the phosphatidylinositol phosphates. No effect of neomycin alone was observed on either glucose uptake or GLUT4myc surface levels (27). Similarly, we did not observe an effect of neomycin treatment alone on the various GLUT4 traffic events examined in this study.

**Immunodetection of GLUT4myc on Plasma Membrane Lawns—**Plasma membrane lawns from L6-myoblasts were prepared by modification developed for 3T3-L1 cells and described above. Cells were serum-starved for 4 h then subjected to the diverse pretreatments or stimuli at 37°C for the indicated time periods (the times chosen were those yielding a maximal response to each stimulus). Cells were then plated on ice, washed twice in ice-cold PBS (pH 7.5) and incubated with ice-cold hypotonic swelling buffer (23 mM KCl, 10 mM HEPES, 5 mM MgCl2, 1 mM EGTA, pH 7.5) for 3 min. Cells were then washed 3 times for 30 s in ice on ice in swelling buffer (230 mM KCl, 30 mM HEPES, 5 mM MgCl2, 3 mM EGTA, 0.5 mM phosphomono-sulfonyl fluoride, 1 mM pepstatin A, 1 mM leupeptin, and 1 mM diithiothreitol, pH 7.5) using a Hert System sonicator (Misonix Inc., Farmingdale, NY). Where no error bars are present, the values were too small to show on the columns (except for conditions assigned a value of 1.0).

**Detection of GLUT4myc C Terminus by Immunoelectron Microscopy—**The method of (30, 31) for immunodetection of GLUT4 on plasma membrane lawns by electron microscopy was used with slight modification. L6 myoblasts differentiated into myotubes on Formvar-coated nickel grids (300 mesh) were serum-deprived, then treated with vehicle (MeSO4 or 100 nM wortmannin for 20 min prior to 100 nM insulin (10 min) at 37°C. Lawns were generated as described above from cells grown on coverslips, fixed with 0.1% glutaraldehyde in PBS for 30 min at room temperature, and blocked with 2% goat serum in PBS. Detection was preceded with C terminus GLUT4 antibody (20 μg/ml) in PBS containing 2% goat serum (60 min), followed by 6 nm gold-conjugated anti-rabbit antibody (1:25 dilution, 60 min). Lawns were fixed with 2% glutaraldehyde for 10 min followed by 1% OsO4 in 0.1 M phosphate buffer (pH 7.4) for 10 min and washed with PO4 buffer three times for 2 min each followed by washing with deH2O for 2 min. Lawns were stained with 2% aqueous uranyl acetate for 10 min prior to dehydration with ethanol. Finally, lawns were dried with a critical point dryer CPD 030 (Bal-Tec, Liechtenstein). Transmission electron microscopy examination was done with a Tecnai 20 at room temperature (FEI, Hillsboro, OR).

**RESULTS**

The experimental premise of this study is that epitopes on different regions of GLUT4myc can be exploited to determine whether GLUT4myc is fully inserted into the plasma membrane, or whether it lies in vesicles adhered/docked to the cytosolic leaflet of the membrane. The myc epitope inserted between transmembrane helices 1 and 2 is expected to face the extracellular milieu when fully fused with the membrane, or the intravesicular space of vesicles docked onto the membrane. Plasma membrane lawns that would retain docked but unfused vesicles would not have the vesicular myc epitope available for antibody decoration unless the docked vesicles are released with detergent once the lawns are generated. Conversely, the last 15 amino acids of the C terminus end or the large cytosolic loop between helices 6 and 7 of the transporter would be available for antibody decoration whether GLUT4myc is inserted into the plasma membrane or present on docked vesicles. The availability of the C terminus or cytosolic antigens for antibody recognition on membrane lawns would, however, be affected by endogenous proteins that might bind to these sites. In this case, high salt or other chaotropic agents that might remove the putative bound proteins would increase C terminus (or cytosol-
surface. Moreover, the follows: All of GLUT4 cells is not affected by such overexpression (8, 26, 35), as mycously determined that GLUT4 compared with muscle or adipose tissue) (34). We have previ-

levels than the endogenous GLUT4 (which is low in these cells

myotubes GLUT4 cells. This behavior is expected given that in L6-GLUT4 myc cells is not affected by such overexpression (8, 26, 35), as follows: All of GLUT4 myc is available for recycling to the cell surface. Moreover, the \( t_{1/2} \) of GLUT4 myc internalization is 3.5 min (similar to that of GLUT4 or IRAP in adipose cells) and

FIG. 1. Distinction of immunofluorescent signals of endogne-

ous GLUT4 and GLUT4 myc C termini through different de-
tector gains. Wild-type L6 myotubes or L6-GLUT4 myc myotubes were grown on coverslips were starved of serum 4 h, then treated without or with 100 nM insulin for 10 min. Plasma membrane lawns were prepared and labeled with monoclonal anti-myc or polyclonal anti-C-terminal GLUT4 antibody, followed by incubation with goat anti-mouse A488, goat anti-rabbit Cy3 antibody, respectively, as described under “Experimental Procedures.” Immunolabeled lawns were processed and analyzed by confocal fluorescence microscopy. Signals were acquired at low and high detector gain levels. At the detector gain used to acquire the C terminus signal of L6-GLUT4 myc myotubes (low gain), there was no detectable signal of the C terminus of wild-type GLUT4 myotubes. The endogenous GLUT4 C terminus of wild-type myotubes was only detectable at the high gain. The results illustrate that the C-terminal signal of the endogenous GLUT4 cannot be detected under the conditions used to acquire the C-terminal signal from the transfected GLUT4 myc. Bar, 10 \( \mu \)m.

FIG. 2. Wortmannin and LY294002 arrest GLUT4 myc vesicle fusion with the membrane. L6-GLUT4 myc myotubes were treated with vehicle (Me2SO) or 100 nM wortmannin (Wm) (A) or 25 \( \mu \)M LY 294002 (B) for 20 min as indicated followed by incubation without (basal) or with 100 nM insulin for 10 min. Plasma membrane lawns were prepared and processed for detection of myc epitope, C-terminal GLUT4, or caveolin by confocal fluorescence microscopy. The fluorescence intensity was quantified from at least 50 plasma membrane lawn pieces for each condition per experiment as described under “Experimental Procedures” and is illustrated as the mean ± fold change per lawn over the basal (± S.E.) obtained from at least five independent experiments. *, \( p < 0.005 \) and #, \( p < 0.05 \) relative to the corresponding basal values (ANOVA).

Wortmannin and LY294002 Arrest GLUT4 myc Vesicle Fu-
sion with the Membrane—Fig. 2A illustrates the gains in myc and C terminus epitopes on membrane lawns derived from basal and insulin-stimulated L6-GLUT4 myc myotubes, quantified as described under “Experimental Procedure.” By comparison, the immunodetection of caveolin on membrane lawns was not affected by insulin stimulation. The plasma membrane content of caveolin was previously shown not to change in response to insulin (37). The gain in myc epitope caused by insulin pretreatment of cells for 10 min was 1.62 ± 0.05-fold relative to the basal value (\( p < 0.005 \)). Wortmannin (100 nM) and LY294002 (25 \( \mu \)M) completely inhibit class IA PI 3-kinase activation (12, 22). Remarkably, pretreatment of myotubes with 100 nM wortmannin abolished the insulin-dependent gain in myc epitope detection while preserving nearly half of the

\[ 2 \] V. Randhawa and A. Klip, unpublished results.
epitopes on plasma membrane lawns from L6 myotubes pretreated with vehicle or 100 nM wortmannin (Wm) for 20 min as indicated, followed by incubation without or with insulin (100 nM for 10 min). Plasma membrane lawns were then labeled and processed for detection of myc or C-terminal epitopes of GLUT4myc by confocal fluorescence microscopy. Where indicated, lawns were treated with 0.1% Triton X-100 after fixation (solid bars). The fluorescence intensity per lawn was quantified from at least 50 plasma membrane lawn pieces for each condition per experiment as described under “Experimental Procedures,” and is expressed as fold change per unit area over the basal (mean ± S.E.) obtained from at least five independent experiments. *, p < 0.005 and #, p < 0.05 relative to the corresponding basal values (ANOVA).

From the above results we hypothesize that 100 nM wortmannin or 25 μM LY294002 abolished the insulin-dependent gain in myc epitope detection on membrane lawns, while preserving significant gain in C terminus epitope (1.40 ± 0.03-fold, p < 0.05 compared with unstimulated cells equally pretreated with LY294002) (Fig. 2B).

To exclude the possibility that the different gains in epitope in plasma membrane lawns from basal or insulin-treated cells compared with basal insulin-stimulated cells with and without insulin stimulation (Fig. 3). Triton X-100 did not affect the detection of either epitope in plasma membrane lawns from basal or insulin-treated cells. However, Triton X-100 revealed an insulin-dependent gain in myc epitope in lawns from cells pretreated with wortmannin plus insulin (1.36 ± 0.01-fold above basal cells pretreated with wortmannin, p < 0.005). By contrast, there was no change in the immunoreactivity of the C terminus upon Triton X-100 treatment of membrane lawns.

Immunoelectron Microscopy Reveals GLUT4 Vesicles on Plasma Membrane Lawns—Collectively, the above results suggest that docked GLUT4myc-containing vesicles are detected in lawns from cells pretreated with wortmannin or LY294002 and stimulated with insulin. To verify this prediction, we explored the presence of docked GLUT4 vesicles on plasma membrane lawns using immunoelectron microscopy. L6 myotubes were pretreated without or with 100 nM wortmannin followed by stimulation without or with insulin. Plasma membrane lawns were generated, and the GLUT4 C terminus was detected by gold-conjugated antibodies and examined by electron microscopy as described in “Experimental Procedures.” Fig. 4 shows representative images of lawns derived from cells of each condition. In lawns from unstimulated cells (whether or not pretreated with wortmannin) as well as in insulin-stimulated cells, GLUT4 was largely detected in flat areas of the membrane, in agreement with previous studies using 3T3-L1 adipocytes (29). Strikingly, however, GLUT4-containing vesicles (~100 nm in diameter) were readily visualized in lawns from cells pretreated with wortmannin prior to insulin stimulation, and there was little immunogold-labeled GLUT4 outside the vesicles. Qualitatively similar results were obtained in five separate experiments analyzing at least six fields in each experiment. These results illustrate the preponderance of GLUT4 in flat areas of the lawns from unstimulated cells (without or with wortmannin) or from insulin-stimulated cells, and a converse preponderance of GLUT4 in vesicles in lawns from wortmannin-pretreated, insulin-stimulated cells.

Insulin Causes Unmasking of the GLUT4myc C Terminus—Further examination of the results in Fig. 2 showed that the gain in C terminus labeling in lawns from insulin-stimulated cells compared with basal cells was significantly larger (2.07 ± 0.11-fold, p < 0.005) than the gain in myc epitope labeling in the same preparations (1.62 ± 0.05-fold, p < 0.005). To exclude the possibility that the different gains in myc and C-terminal signals might be due to difference in affinity of each antibody for its epitope or to characteristics of the fluorophores used, various combinations of primary and secondary antibodies were used. Table I shows that the insulin-dependent gain in fluorescence did not depend on the nature of the antibody species or the fluorophore attached to the secondary antibody. In addition, the gain in myc epitope remained 1.62 ± 0.21-fold relative to basal (p < 0.05) and that in C terminus remained 2.00 ± 0.19-fold relative to basal (p < 0.005, n = 5), when the concentration of primary antibodies was used, suggesting that the differences were not due to lack of epitope saturation.

The antigenic availability of the large cytosolic loop of
GLUT4myc was also tested. Insulin caused a gain in immunofluorescent signal of this epitope of 2.01 ± 0.16-fold relative to basal (p < 0.005), which was comparable to the gain in signal of the C terminus epitope. In contrast, an antibody raised to the first exofacial loop of GLUT4 detected a 1.65 ± 0.15-fold gain in signal on membrane lawns (p < 0.005), akin to the gain in myc epitope signal. These observations corroborate that the gain in lawn-associated fluorescent signal is larger for cytosolic than for exofacial epitopes of GLUT4.

The gain in antigenicity of the C terminus relative to the myc epitope observed in lawns was also evident in whole cells. L6-GLUT4myc or wild-type myoblasts were permeabilized with Triton X-100 after fixation (see “Experimental Procedures”). The whole cell signal detected by the polyclonal anti-C terminus antibody was elevated by 27% in response to insulin when serum-starved for 4 h, then stimulated with 100 nM insulin for 10 min. As with GLUT4

- Myoblast monolayers of parental L6 cells or GLUT4myc cells were serum-starved for 4 h, then stimulated with 100 nM insulin for 10 min. The myc or C-terminal epitopes were detected by immunofluorescence after cell permeabilization with 0.1 % Triton X-100, and quantification was performed as described under “Experimental Procedures.” Results from five independent experiments are expressed as the mean ± S.E. relative to the corresponding unstimulated cells (a value of 1).

**TABLE I**

| Primary antibody | Secondary antibody | Fluorescence intensity over basal |
|------------------|--------------------|----------------------------------|
| Monoclonal anti-Myc | Alexa 488-conjugated goat anti-mouse IgG | 1.26 ± 0.05* |
|                  | Cy3-conjugated goat anti-mouse IgG   | 1.64 ± 0.08* |
| Polyclonal anti-Myc | Alexa 488-conjugated goat anti-rabbit IgG | 1.63 ± 0.10* |
| Polyclonal anti-exofacial loop | Alexa 488-conjugated rabbit anti-goat IgG | 1.63 ± 0.06* |
|                  | Cy3-conjugated donkey anti-goat IgG  | 1.65 ± 0.15* |
| Polyclonal anti-C terminus | Alexa 488-conjugated goat anti-rabbit IgG | 2.00 ± 0.12* |
|                  | Cy3-conjugated goat anti-rabbit IgG  | 2.07 ± 0.11* |
| Polyclonal anti-cytoplasmic loop | Alexa 488-conjugated goat anti-rabbit IgG | 2.01 ± 0.16* |

* p < 0.005 relative to the corresponding basal value (Student’s t test).

**TABLE II**

| Insulin-induced gain in GLUT4 C terminus epitope | α-myc | α C terminus |
|--------------------------------------------------|-------|-------------|
| L6-wild-type cells                                | —     | 1.27 ± 0.05* |
| L6-GLUT4myc cells                                | 0.98 ± 0.03 | 1.26 ± 0.06* |

* p < 0.005 relative to the corresponding basal values (Student’s t test).

**FIG. 5.** High salt treatment of plasma membrane lawns increases GLUT4 C terminus antigenicity in the basal state. L6-GLUT4myc myotubes were incubated without or with insulin (100 nM for 10 min), as indicated. Membrane lawns were generated then incubated with PBS without or with an additional 150 or 500 mM NaCl for 7 min on ice prior to fixation, epitope labeling, and confocal fluorescence microscopy analysis. Results are the -fold change per unit area over the basal (mean ± S.E.) obtained from at least four independent experiments. *, p < 0.005; #, p < 0.05 relative to the corresponding basal values (ANOVA).

As with GLUT4myc myotubes, the C-terminal antigenicity of high salt wash (results not shown), suggesting that fixation prevented the removal of the putative masking protein.
endogenous GLUT4 in wild-type, unstimulated L6 myotubes was also elevated upon treatment of the corresponding lawns with PBS supplemented with 500 mM NaCl (to a value of 1.26 ± 0.04-fold above the untreated control, p < 0.005, n = 3, t test). Insulin caused a gain in endogenous C terminus of 1.81 ± 0.05-fold above basal control (p < 0.005), and this gain was not affected by high salt treatment of the lawns (remaining at 1.88 ± 0.04-fold of above basal control; p < 0.005, n = 3).

The above results support the possibility that the larger gain in lawn-associated C terminus than myc signals observed in response to insulin arises from unmasking of the C terminus, which is hindered from antibody recognition in the basal state. However, it is also possible that the myc epitope becomes antigenically compromised in the insulin-stimulated state. The following experiment with a GLUT4myc-eGFP chimera addresses this possibility. Presumably, the putative C terminus unmasking of GLUT4 would not affect the signal of eGFP.

PI(3,4,5)P3 and PI3P Increase GLUT4 Presence by the Membrane but Only PI(3,4,5)P3 Causes GLUT4 Fusion—The major product of class IA PI 3-kinase is PI(3,4,5)P3, and this lipid is elevated in response to insulin in L6 myotubes (39). We have recently shown that exogenous administration of PI(3,4,5)P3 (delivered via coupling with cationic carrier) suffices to cause significant GLUT4myc arrival at and fusion with the plasma membrane in intact L6 myoblasts, without increasing glucose uptake (27). It was therefore important to assess whether GLUT4myc membrane insertion in response to PI(3,4,5)P3 is detectable in the lawns, and subsequently to determine whether this phosphoinositide can also cause unmasking of the GLUT4 C terminus. In addition to PI(3,4,5)P3, PI3P is produced by muscle and fat cells in response to insulin (40). Therefore, we compared the effect of the two phosphoinositides on GLUT4 arrival at the membrane, and on the availability of the myc epitope for antigenic recognition. L6 myotubes were incubated with PI(3,4,5)P3 or PI3P and carrier (10 μM each) for 20 min, and membrane lawns were generated and labeled with monoclonal anti-myc and secondary antibody. Consistent with our published results in intact myoblasts (27), carrier-mediated deliveries of PI3P, of PI(4,5)P2 or of carrier alone, failed to change the lawn levels of myc epitope. The results using 10 μM concentrations of each agent were as follows: 1.04 ± 0.23, 0.99 ± 0.09-, and 0.99 ± 0.20-fold above untreated controls, respectively. No effects were seen either using 20 μM concentrations of the carrier and PI3P or PI(4,5)P2, as indicated. Plasma membrane lawns were generated and fixed, labeled with monoclonal anti-myc antibody followed by goat anti-mouse A488 antibody (A) or polyclonal anti-C terminus of GLUT4 antibody followed by goat anti-rabbit Cy3 (B) and processed for confocal fluorescence microscopy. Where indicated, lawns were treated with 0.1% Triton X-100 after fixation (solid bars). The fluorescence intensity per lawn was quantified from at least 50 plasma membrane lawn pieces for each condition per experiment as described under “Experimental Procedures.” Results are the mean ± fold change per unit area over the basal (± S.E.) obtained from at least five independent experiments. * p < 0.005; #, p < 0.05 relative to the corresponding basal values (ANOVA).

The above results suggest that PI3P may have not mobilized GLUT4myc to the membrane at all, or that GLUT4myc-containing vesicles may be docked but unfused, keeping the myc epitope unavailable for antigenic recognition. We therefore treated the plasma membrane lawns with 0.1% Triton X-100 after fixation, prior to myc immunodetection (Fig. 6A). This treatment revealed a PI3P-dependent gain of previously latent myc epitopes (1.40 ± 0.02-fold relative to untreated controls, p < 0.05), presumably present in docked, unfused vesicles. In contrast, permeabilizing the lawns with Triton X-100 barely affected the gain in myc signal observed in intact lawns from
cells pretreated with PI(3,4,5)P$_3$ (1.62 $\pm$ 0.04-fold relative to untreated controls, $p < 0.005$). The myc signal on lawns from cells pretreated with PI(4,5)P$_2$ plus carrier or carrier alone was not affected by Triton-X100 treatment (0.93 $\pm$ 0.12- and 0.99 $\pm$ 0.11-fold relative to untreated controls). The above results suggest that both PI(3,4,5)P$_3$ and PI3P increase the presence of GLUT4 at the cell periphery, whereas PI(3,4,5)P$_2$ leads to GLUT4 vesicle fusion with the plasma membrane, P3P does not.

**P3P, but Not PI(3,4,5)P$_3$, Promotes GLUT4 C Termi

Following the results described above, it was of interest to examine the combined effect of PI(3,4,5)P$_3$ and PI3P on GLUT4/myc (10 $\mu$m each). This treatment produced a gain in myc epitope on membrane lawns of 1.65 $\pm$ 0.08-fold above basal, $p < 0.005$, and a gain in C terminus of 1.80 $\pm$ 0.08, $p < 0.005$. Hence, although the myc epitope did not increase significantly under these conditions compared with the effect of PI(3,4,5)P$_3$ alone, the gain in C terminus signal was augmented by the presence of P3P. These results are consistent with the possibility that P3P causes unmasking of the C terminus of GLUT4/myc molecules recruited by PI(3,4,5)P$_3$. Moreover, PI(3,4,5)P$_3$ added along with insulin did not affect the insulin-dependent gain in C terminus (results not shown), suggesting that PI(3,4,5)P$_3$ itself does not mask this epitope.

Finally, as presented in Table III, in cells with class IA PI 3-kinase inhibited by 100 nm wortmannin, addition of exogenous PI(3,4,5)P$_3$ was still able to cause GLUT4 translocation to the plasma membrane (middle column). In cells pretreated with 100 nm wortmannin and stimulated with insulin, PI(3,4,5)P$_3$ caused GLUT4 recruitment and this GLUT4 underwent C-terminal unmasking (third column), presumably through P3P produced in response to insulin.

| Antibody | Lawn associated antibody (fold of corresponding basal) | Wm + I | Wm + PI(3,4,5)P$_3$ | Wm + I + PI(3,4,5)P$_3$
|---|---|---|---|---|
| α-myc | 1.00 $\pm$ 0.03 | 1.57 $\pm$ 0.12 | 1.61 $\pm$ 0.09 |
| α C terminus | 1.41 $\pm$ 0.04 | 1.15 $\pm$ 0.05 | 1.70 $\pm$ 0.02 |

* $p < 0.05$ compared with the C terminus gain on membrane lawns from cells treated with wortmannin prior to insulin stimulation (ANOVA). The myc and C terminus signals of unstimulated cells were not affected by wortmannin only treatment (not shown).

GLUT4/myc Fusion and Unmasking in Routed-up L6 Myo

---The proposed three stages in GLUT4 translocation were also analyzed in rounded-up L6 myoblasts. This preparation enables one to label the surface-exposed myc epitope in intact cells, or the intravesicular myc epitope and cytosolic-facing C terminus in permeabilized cells. Hence it offers the possibility to confirm the observations made in lawns without resorting to sonication. Fig. 5A illustrates that insulin caused a gain in myc epitope at the surface of intact cells, which was fully prevented by pretreatment with 100 nm wortmannin. Cellular permeabilization with Triton X-100 reveals the intracellular depots of GLUT4/myc, whether perinuclear or at the cell periphery. Such permeabilization enabled detection of myc epitope along the periphery in wortmannin-treated, insulin-stimulated cells (Fig. 8B), despite the lack of gain in myc signal in intact cells. Quantitative analysis was performed as described under "Experimental Procedures," and the results are illustrated in Fig. 8C. These results corroborate the observations made in membrane lawns suggesting that unfused vesicles are arrested at the membrane under these conditions. PI(3,4,5)P$_3$ also provoked a significant gain in surface myc epitope in intact myoblasts, suggesting that PI(3,4,5)P$_3$ alone can mobilize GLUT4 toward the membrane. The peripheral
response to PI(3,4,5)P₃ was inaccessible to detection by anti-C-terminal antibodies in permeabilized cells, cementing the notion that this phosphoinositide suffices to cause arrival and fusion but not unmasking of GLUT4 at the cell surface. The gain in C terminus epitope at the periphery of PI3P-treated myoblasts is further consistent with the results using plasma membrane lawns suggesting that, like insulin, PI3P causes GLUT4 C terminus unmasking.

**DISCUSSION**

**A Model for GLUT4 Arrival, Fusion, and C Terminus Unmasking**—The results presented here support a model whereby insulin causes arrival, membrane fusion, and the removal of a putative masking protein from GLUT4. Although it is well documented that class IA PI 3-kinase is activated by insulin and that dominant-negative mutants of the enzyme prevent the gain in surface GLUT4, the precise step(s) in GLUT4 cycling regulated by the enzyme remained to be defined. Here we establish a strategy to determine the degree of incorporation of GLUT4 with the plasma membrane, by following simultaneously the changes in antigenic availability of exofacially and endofacially facing epitopes on the transporter. The myc, C-terminal, and middle loop epitopes of GLUT4myc should change in parallel unless there are constraints to the recognition of either one. Under the fluorescence acquisition conditions used the contribution of endogenous GLUT4 is negligible, and therefore only GLUT4myc is detected by the antibodies used. Membrane lawns were used as a platform containing membrane-inserted and membrane-attached vesicles that is amenable to quantitative fluorescence assessment, and rounded-up myoblasts, intact or permeabilized, are used to visualize inserted from docked GLUT4, respectively.

**GLUT4 Fusion with the Membrane**—The first proposition of this study is that class IA PI 3-kinase input regulates the fusion of insulin-sensitive, GLUT4myc-containing vesicles with the plasma membrane. This possibility is raised by three complementary observations as follows: (a) In intact, rounded-up myoblasts, 100 nM wortmannin and 25 μM LY294002 (result not shown) prevent the insulin-induced gain in myc epitope availability from the extracellular milieu, yet, upon permeabilization, the gain in GLUT4 C terminus is readily visualized in the periphery of the cell. (b) In plasma membrane lawns derived from wortmannin- or LY294002-pretreated cells, there was no insulin-dependent gain in antigenically available myc epitope, although, as above, a substantial gain in C terminus was detected. (c) Lawn permeabilization with Triton X-100, expected to permeabilize any vesicular bodies present on the lawns, exposed significant myc epitope to match the insulin-dependent gain in C terminus. Consistent with this observation, vesicles containing GLUT4 were detected by immunoelectron microscopy on lawns from wortmannin-pretreated, insulin-stimulated cells.

We interpret these results to indicate that, at the concentrations used, wortmannin (100 nM) or LY294002 (25 μM) allow significant arrival of GLUT4 at the vicinity of the plasma membrane but prevent the fusion of GLUT4 vesicles. With one exception (41), all previous studies in the literature examining the role of PI 3-kinase in GLUT4 translocation used only high concentrations of wortmannin (300 to 1000 nM) followed by insulin stimulation and immunofluorescent detection of membrane GLUT4 via its C terminus (42–44) and failed to detect any gain in membrane GLUT4 under these conditions. Indeed, we observed that the insulin-dependent gain in C terminus availability declined gradually in lawns from cells pretreated with higher concentrations of wortmannin (down to 1.2 ± 0.3-fold gain in the presence of 1 μM wortmannin). High concen...
trations of LY294002 also virtually eliminated the gain in C terminus epitope on the lawns from insulin-stimulated cells (1.14 ± 0.09-fold gain in the presence of 75 μM LY294002). Collectively, these observations suggest that migration of GLUT4 toward the plasma membrane (the actual translocation) is less sensitive to these inhibitors than the insertion of GLUT4 into the membrane. When this study was being completed, Bose et al. (45) reported that the fluorescence of GLUT4myc-GFP transfected into 3T3-L1 adipocytes increased in cells pretreated with LY294002 and insulin, yet the myc epitope was unavailable from the cell exterior. Similarly, van Dam et al. (46) reported that Akt activation correlates with GLUT4 insertion rather than arrival at the membrane. Our study expands these observations by demonstrating that the peripheral myc epitope is trapped in Triton X-100-sensitive structures, likely docked but unfused GLUT4 vesicles. Indeed, using immunoelectron microscopy we directly visualize GLUT4-containing vesicles on the lawns of wortmannin-pretreated, insulin-stimulated cells. To our knowledge, this is the first visualization of GLUT4 vesicles adhered to the plasma membrane. The fact that GLUT4 is found in membrane-adhered vesicles in the presence of wortmannin suggests that fusion is a regulated step. Moreover, because no membrane-adhered vesicles were observed in the absence of wortmannin, GLUT4 arrival rather than fusion appears to be the rate-limiting step in basal and insulin-stimulated cells.

Unmasking of the GLUT4 C Terminus—The second major observation of this study is that the insulin-dependent gain in myc epitope detected on membrane lawns is routinely and significantly lower than the gain in C terminus detection. The difference holds irrespective of the nature of the antibodies used to detect each epitope (monoclonal or polyclonal) or of the type of fluorophore associated with the secondary antibody. The gain in myc signal was, however, comparable to that of an exofacial epitope between transmembrane helices 1 and 2 prior to the myc epitope insert in GLUT4myc, whereas the gain in C terminus was comparable to that of the middle cytosolic loop of the transporter. We interpret these results to indicate that either the exofacial epitopes are less available for antigenic recognition in the insulin-stimulated state than in the basal state relative to the cytosolic ones or that the cytosolic epitopes are less available for recognition in the basal state. Several observations support the latter possibility, as follows: (a) Treatment of lawns with Triton X-100, shown above to permeabilize docked vesicles, failed to increase the gain in myc signal, suggesting that the lesser myc signal is not due to unavailability due to an abundance of docked, unfused vesicles in the insulin-stimulated state. (b) Treatment of lawns with high salt solutions increased the C-terminal antigenicity in the basal, but not in the insulin stimulated state, suggesting that a C terminus obstructing protein might have been removed from the lawns of basal-state cells. This change was precluded if the lawns were fixed with PFA prior to exposure to high salt. (c) The insulin-dependent gain in myc epitope on lawns was similar to that in eGFP signal, analyzed in cells transiently transfected with GLUT4myc-eGFP. (d) Conversely, the C terminus signal was of similar magnitude in GLUT4myc-overexpressing myotubes as in lawns from L6-wild-type myotubes, and high salt treatment of lawns also increased the basal-state signal of endogenous GLUT4. (e) As in membrane lawns, the C-terminal signal from the entire cell was elevated by insulin treatment. In contrast, the myc epitope signal of the entire cell remained unaffected by the treatment with the hormone.

These results are consistent with the possibility that a so far undefined protein that partially obstructs cytosolic epitopes from recognition by antibodies might be released in response to insulin. Indeed, two separate studies have reported insulin-dependent increased reactivity of the C terminus of GLUT4 in adipocytes (47) and skeletal muscle (48), presumably as a result of removal of an interfering protein or a conformational change of the C terminus. It remains possible that the increase in C terminus immunoreactivity may be elicited by a conformational change in GLUT4. Such conformational change would have to be induced by insulin stimulation and reproduced by the high salt treatment of lawns. Several proteins can bind to the C terminus of GLUT4 (49–51), but so far none of these was shown to be sensitive to insulin stimulation. On the other hand, it was recently reported that the protein TUG binds to GLUT4 (52), and this binding is diminished in response to the hormone (52). Future work should investigate the nature of the protein purported to determine antigenic availability of cytosolic epitopes on GLUT4.

Differential Regulation of GLUT4 Fusion and Unmasking by Distinct Phosphoinositides—The third major observation of this study is that PI(3,4,5)P3 and PI3P delivery each promotes significant arrival of GLUT4myc at the cell periphery. These results, along with the relative insensitivity to 100 nM wortmannin of GLUT4myc arrival at the cell periphery, raise the possibility that the class IA PI 3-kinase product PI(3,4,5)P3 and the class II PI 3-kinase C2α product PI3P might be jointly responsible for the insulin-dependent arrival of GLUT4myc at the vicinity of the plasma membrane. However, whereas PI(3,4,5)P3 causes GLUT4myc externalization, presumably via vesicle fusion with the membrane, the GLUT4myc molecules summoned to the periphery by addition of PI3P do not result in productive externalization. We surmise that only PI(3,4,5)P3 but not PI3P can cause GLUT4myc-vesicle fusion with the membrane. Recently, Maffucci et al. (40) also reported that carrier delivery of PI3P promotes GLUT4 arrival at periphery of the cells but interpreted this result to be equivalent to GLUT4 insertion. Our approach comparing detection of the C terminus and the myc epitope on GLUT4myc affords the distinction between these two steps and reveals that each phosphoinositide has distinct actions on GLUT4 externalization. Similarly, Chaussade et al. (53) recently reported that overexpression of myotubulin, a PI3P phosphatase, impaired insulin-induced GLUT4 translocation to the plasma membrane probably because of reduced PI3P production by insulin in 3T3-L1 adipocytes. As in the study of Maffucci et al., GLUT4-GFP was used, precluding differentiation between adhered and fused GLUT4-vesicles at the plasma membrane. In those studies, as in the present case, the effect of phosphatidylinositol phosphates was determined on the steady-state levels of surface GLUT4 and not on the individual rates of exocytosis or endocytosis. It is unlikely that PI(3,4,5)P3 increased surface GLUT4 levels by reducing the internalization of pre-existing GLUT4 molecules. This is because preventing GLUT4myc internalization should have elevated the myc and C-terminal signals by the same proportion, yet PI(3,4,5)P3 only increased the myc signal. However, it is theoretically possible that PI3P reduces the progression of membrane-attached coated vesicles containing GLUT4 toward the early endosome. These interesting possibilities must be addressed in the future.

Importantly, while PI(3,4,5)P3 suffices to promote fusion of the GLUT4-containing vesicles arriving at the plasma membrane, it does not emulate the C-terminal unmasking provoked by PI3P and insulin in these muscle cells. In contrast, in lawns of 3T3-L1 adipocytes treated with PI(3,4,5)P3 we observed a gain in GLUT4 C terminus availability (27), but it
size that some combination of these two phosphoinositides contributes to the insulin-dependent regulation of all three events: arrival, fusion, and unmasking.

Acknowledgments—We thank Dr. P. J. Bilan for careful reading of the manuscript and Robert Temkin for kind help with electron microscopy.

REFERENCES

1. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758–4762
2. Suzuki, K., and Kono, T. (1986) Proc. Natl. Acad. Sci. U. S. A. 77, 2542–2545
3. Klip, A., Ramal, T., Young, D. A., and Holloszy, J. O. (1987) FEBS Lett. 224, 224–230
4. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) Nature 333, 183–185
5. Birnbaum, M. J. (1989) Cell 57, 305–315
6. Deen, A. G., Ramal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., Holloszy, J. O., and Klip, A. (1996) J. Biol. Chem. 261, 13427–13430
7. Sato, S., Nishimura, H., Clark, A. E., Kozka, J. L., Vannucci, S. J., Simpson, I. A., Quon, M. J., Cushman, S. W., and Holman, G. D. (1993) J. Biol. Chem. 268, 17820–17829
8. Li, D., Randhawa, V. K., Patel, N., Hayashi, M., and Klip, A. (2001) J. Biol. Chem. 276, 22283–22291
9. Kahn, B. B., Simpson, I. A., and Cushman, S. W. (1988) J. Clin. Invest. 82, 691–699
10. Klip, A., Ramal, T., Bilan, P. J., Cartee, G. D., Guerre, R., and Holloszy, J. O. (1990) Biochem. Biophys. Res. Commun. 172, 728–736
11. Zierath, J. R., He, L., Guma, A., Odegoard Wahlstrom, E., Klip, A., and Waterfield, M. D. (1996) Diabetes 45, 1180–1189
12. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
13. Tsakiridis, T., Vranic, M., and Klip, A. (1995) Biochem. J. 309, 1–5
14. Wang, Q., Somwar, R., Ristroph, P. J., L. J. Z., Jin, J., Woodgett, J. R., and Klip, A. (1999) Mol. Cell. Biol. 19, 4008–4018
15. Tengholm, A., and Meyer, T. (2002) Curr. Biol. 12, 1871–1876
16. Foster, L. J., Li, D., Randhawa, V. K., and Klip, A. (2001) J. Biol. Chem. 276, 44212–44221
17. Khayat, Z. A., Tong, P., Yaworsky, K., Bloch, R. J., and Klip, A. (2000) J. Cell Biol. 151, 279–289
18. Patel, N., Rudich, A., Khayat, Z. A., Garg, R., and Klip, A. (2003) Mol. Cell. Biol. 23, 4613–4626
19. Brown, R. A., Domin, J., Arcaro, A., Waterfield, M. D., and Shepherd, P. R. (1999) J. Biol. Chem. 274, 14529–14532
20. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 67, 481–507
21. Rosenman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1411–1415
22. Domin, J., Pages, F., Volinia, S., Rittenhouse, S. E., Zvelebil, M. J., Stein, R. C., and Waterfield, M. D. (1997) Biochem. J. 326, 139–147
23. Sergeant, R. J., and Paquet, M. R. (1993) Mol. Biochem. Pathol. 90, 913–919
24. Shigematsu, S., Khan, A. H., Kanakizaki, M., and Pessin, J. E. (2002) Mol. Endocrinol. 16, 1060–1068
25. Kishi, K., Muramoto, N., Nakaya, Y., Miyata, I., Hagi, A., Hayashi, H., and Ebina, Y. (1998) Diabetes 47, 550–558
26. Ueyama, A., Yaworsky, K. L., Wang, Q., Ebina, Y., and Klip, A. (1999) Am. J. Physiol. 275, E572–E578
27. Sweeney, G., Garg, R. R., Tokida, R. I., Li, D., Ishiki, M., Somwar, R., Foster, L., Neilsen, P. O., Prestwich, G. D., Rudich, A., and Klip, A. (2004) J. Biol. Chem. 279, 32233–32242
28. Sweeney, G., Somwar, R., Ramal, T., Volchuk, A., Ueyama, Y., and Klip, A. (1999) J. Biol. Chem. 274, 10071–10078
29. Robinson, L. J., Pang, S., Harris, D. S., Heuser, J., and James, D. E. (1992) J. Cell Biol. 117, 1181–1186
30. Parpal, S., Karlsson, M., Thorn, H., and Stralfors, P. (2001) J. Biol. Chem. 276, 9670–9678
31. Karlsson, M., Thorn, H., Parpal, S., Stralfors, P., and Gustavsson, J. (2002) Pfluegers J. 16, 249–253
32. Zierath, J. R., Krook, A., and Wallberg-Henriksson, H. (1998) Mol. Cell. Biochem. 182, 153–160
33. Randhawa, V. K., Thong, F. S., Lim, D. Y., Li, D., Garg, R. R., Rudge, R., Gali, T., Rudich, A., and Klip, A. (2004) Mol. Cell. Biochem. 255, 5565–5573
34. Huang, C., Somwar, R., Patel, N., Niu, W., Tokor, D., and Klip, A. (2002) Diabetes 51, 2090–2098
35. Rudich, A., Konrad, D., Tokor, D., Ben-Romano, R., Huang, C., Niu, W., Garg, R. R., Wijesekara, N., Germinario, R. J., Bilan, P. J., and Klip, A. (2003) Diabetologia 46, 649–658
36. Zeigerer, A., Lampson, M. A., Kalyowski, O., Sabatini, D. D., Adesnik, M., Ren, M., and McGraw, T. E. (2002) Mol. Cell. Biol. 22, 4243–4255
37. Teruel, T., Hernandez, R., and Lorenzo, M. (2001) Diabetes 50, 2563–2571
38. Mozzarelli, G. F., Niederberger, P., and Hutter, R. (1978) Anal. Biochem. 80, 220–233
39. Tsakiridis, T., McDowell, H. E., Walker, T., Downes, C. P., Hundal, H. S., Vranic, M., and Klip, A. (1995) Endocrinology 136, 4315–4322
40. Maffucci, T., Brancaccio, A., Piccolo, E., Stein, R. C., and Palasch, M. (2003) EMBO J. 22, 4178–4189
41. James, D. J., Salaun, C., Brandie, F. M., Connell, J. M., and Chamberlain, L. H. (2004) J. Biol. Chem. 279, 20567–20570
42. Le Marchand-Brustel, Y., Gautier, N., Cormont, M., and Van Obberghen, E. (1995) Endocrinology 136, 3564–3570
43. Egawa, K., Sharma, P. M., Nakashima, N., Huang, Y., Huer, E., Boss, G. R., and Olefsky, J. M. (1999) J. Biol. Chem. 274, 14306–14314
44. Imamura, T., Ishihashi, K., Dalle, S., Ugi, S., and Olefsky, J. M. (1999) J. Biol. Chem. 274, 33691–33695
45. Bose, A., Robida, S., Fuciniitti, P. S., Chawla, A., Fogarty, K., Corvera, S., and Czech, M. P. (2004) Mol. Cell. Biol. 24, 5447–5458
46. van Dam, E. M., Govers, R., and James, D. E. (2005) Mol. Endocrinol. 19, 1067–1077
47. Smith, R. M., Charron, M. J., Shah, N., Lodish, H. F., and Jarett, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6893–6897
48. Wang, W., Hansen, P. A., Marshall, B. A., Holloszy, J. O., and Mueckler, M. (1996) J. Cell Biol. 135, 415–430
49. Kao, A. W., Noda, Y., Johnson, J. H., Pessin, J. E., and Saltiel, A. R. (1999) J. Biol. Chem. 274, 17742–17747
50. Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K. C., and Smith, R. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1125–1130
51. Lalioti, V. S., Vergaraigua, S., Pulido, D., and Sandoval, I. V. (2002) J. Biol. Chem. 277, 19785–19791
52. Bogan, J. S., Hendin, N., McKee, A. E., Tsao, T. S., and Lodish, H. F. (2003) Nature 425, 727–733
53. Chauvade, C., Pirola, L., Bonnafous, S., Blondeau, F., Brenz-Vera, S., Tronchere, H., Portis, F., Ruscioni, S., Payastra, B., Laporte, J., and Van Obberghen, E. (2003) Mol. Endocrinol. 17, 2445–2460
54. Malide, D., Ramm, G., Cushman, S. W., and Slot, J. W. (2000) J. Cell Sci. 113, 4203–4210
Insulin Regulates the Membrane Arrival, Fusion, and C-terminal Unmasking of Glucose Transporter-4 via Distinct Phosphoinositides

Manabu Ishiki, Varinder K. Randhawa, Vincent Poon, Lellean JeBailey and Amira Klip

J. Biol. Chem. 2005, 280:28792-28802.
doi: 10.1074/jbc.M500501200 originally published online June 13, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500501200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 35 of which can be accessed free at http://www.jbc.org/content/280/31/28792.full.html#ref-list-1