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# Differential effects of phosphatidylinositol 3-kinase inhibition on intracellular signals regulating GLUT4 translocation and glucose transport

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**Summary**

Phosphatidylinositol (PI) 3-kinase is required for insulin-stimulated translocation of GLUT4 to the surface of muscle and fat cells. Recent evidence suggests that the full stimulation of glucose uptake by insulin also requires activation of GLUT4, possibly via a p38 mitogen-activated protein kinase (p38 MAPK)-dependent pathway. Here we used L6 myotubes expressing myc-tagged GLUT4 to examine at what level the signals regulating GLUT4 translocation and activation bifurcate. We compared the sensitivity of each process, as well as of signals leading to GLUT4 translocation (Akt and atypical protein kinase C) to PI 3-kinase inhibition. Wortmannin inhibited insulin-stimulated glucose uptake with an $IC_{50}$ of 3 nM. In contrast, GLUT4myc appearance at the cell surface was less sensitive to inhibition ($IC_{50}$ = 43 nM). This dissociation between insulin-stimulated glucose uptake and GLUT4myc translocation was not observed with LY294002 ($IC_{50}$ = 8 and 10 μM, respectively). The sensitivity of insulin-stimulated activation of PKCζ/λ and Akt1, Akt2 and Akt3 to wortmannin ($IC_{50}$ = 24, 30, 35 and 60 nM, respectively) correlated closely with inhibition of GLUT4 translocation. In contrast, insulin-dependent p38 MAPK phosphorylation was efficiently reduced in cells pretreated with wortmannin, with an $IC_{50}$ of 7 nM. Insulin-dependent p38α and p38β MAPK activities were also markedly reduced by wortmannin ($IC_{50}$ = 6 and 2 nM, respectively). LY294002 or transient expression of a dominant inhibitory PI 3-kinase construct (Δp85) however, did not affect p38 MAPK phosphorylation. These results uncover a striking correlation between PI 3-kinase, Akt, PKCζ/λ and GLUT4 translocation on one hand, and their segregation from glucose uptake and p38 MAPK activation on the other, based on their wortmannin sensitivity. We propose that a distinct, high affinity target of wortmannin, other than PI 3-kinase, may be necessary for activation of p38 MAPK and GLUT4 in response to insulin.

## Introduction

It has been known for 20 years that insulin causes recruitment of glucose transporters (GLUTs)[1] to the surface of muscle and fat cells (1). However, numerous studies have concluded that the increase in the cell surface content of the muscle/fat-specific GLUT4 does not correlate quantitatively with the degree of stimulation of glucose uptake. This disparity between the magnitude of GLUT4 translocation and the stimulation of glucose uptake has been observed in mature skeletal muscle (2-5), primary adipocytes (6-8) and muscle and fat cell lines (9,10). Recently, we developed a muscle cell line overexpressing GLUT4 fused to a myc epitope that becomes exposed at the cell surface, allowing for the detection of GLUT4 translocation in intact cells (11,12). Using this system, we reported that GLUT4 translocation precedes the stimulation of glucose uptake by at least 2 min (13). In addition, we have identified conditions in which insulin-dependent stimulation of glucose uptake can be reduced in the face of intact GLUT4 translocation (9,14). These conditions include diverse inhibitors of p38 mitogen-activated protein kinase (p38 MAPK) (9,14), low temperature (13) and leptin (15). Conversely, other studies have shown that glucose uptake can be augmented in 3T3-L1 adipocytes by insulin while GLUT4 translocation is completely inhibited (16). Similarly, protein synthesis inhibitors elevate glucose uptake in 3T3-L1 adipocytes without any significant gain in cell surface GLUT4 content (17,18). Taken together, these studies have suggested that at least two events culminate in the stimulation of glucose uptake: translocation and activation of GLUT4. We have further proposed that p38 MAPK may be an integral component of the signalling pathway regulating GLUT4 activity (9,13,14,19).

It is well established that the activity of the lipid kinase phosphatidylinositol (PI)-kinase is necessary for GLUT4 translocation to the plasma membrane in muscle and fat tissues and

cells, based on the use of pharmacological inhibitors (20-24), expression of dominant-negative mutants of type 1 PI 3-kinase (25-27) or microinjection of PI 3-kinase neutralizing antibodies (10). Two downstream effectors of PI 3-kinase, the serine/threonine kinases Akt and atypical protein kinase C appear to relay the signals required for GLUT4 translocation (28-30).

It was recently demonstrated that glucose uptake in 3T3-L1 adipocytes was reduced by concentrations of wortmannin that do not affect GLUT4 translocation (10). These results raise the question of where the signals regulating GLUT4 translocation and activation bifurcate. Answering this question was the objective of the present study. Using L6 myotubes expressing GLUT4myc we determined the sensitivity of glucose uptake, GLUT4 translocation, and the signals thought to regulate these two parameters to wortmannin and LY294002. Strikingly, we found that very low concentrations of wortmannin, but not LY294002 or dominant negative PI 3-kinase (Δp85), prevented p38α and p38β MAPK activation by insulin. The stimulation of glucose uptake was also exquisitely susceptible to inhibition by wortmannin. In contrast, inhibition of Akt or PKCζ/λ activity by LY294002 and/or wortmannin correlated more closely with GLUT4 translocation.

## Experimental Procedures

**Materials.** ATF2 fusion protein, phospho-specific antibodies to Akt (T308 or S473) and p38 MAPK (T180 and Y182), monoclonal phospho-specific anti-p38 MAPK antidbody conjugated to agarose beads and antibodies to p38α MAPK (used to measured kinase activity) were purchased from New England Biolabs (Beverly, Ma). Antibodies to Akt1 (D-17), phosphotyrosine (PY99), p38α and p38β MAPK, c-myc (9E10) and PKCζ/λ (C-20) were from Santa Cruz Biotechnology (Santa Cruz, Ca). Antibodies to Akt2, Akt3 and IRS-1, Crosstide (9 amino acid Akt substrate derived from GSK-3) and myelin basic protein were from Upstate Biotechnology Inc (Lake Placid, NY). O-phenylenediamine dihydrochloride was obtained from Sigma (St Louis, MO).

**Cell Culture and transfection of L6-GLUT4myc cells.** GLUT4myc cDNA was constructed by inserting the human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 and subcloned into the pCXN2 vector (31). The plasmid was stably transfected into L6 myoblasts (32). L6-GLUT4myc myoblasts were maintained in minimal essential medium-α supplemented with 10% FBS in a humidified atmosphere of air and 5% $CO_2$ at 37ºC. Myoblasts were differentiated in medium supplemented with 2% FBS. Glucose uptake and GLUT4myc translocation were determined in cells grown in 24-well plates (1 cm diameter). Cells were grown in 6- and 12-well (2.5 cm diameter) plates for determination of kinase activities and protein phosphorylation, respectively. Cells were seeded at a density of 40 000 cells/well on glass coverslips for immunofluorescence. Transfection was performed according to the Effectene product manual (Qiagen) and is described in details elsewhere (25). The construct pSG5p85αΔSH2, referred to as Δp85, the dominant-negative mutant of Type I PI 3-kinase (33),

was a gift from Dr. Julian Downward (Imperial Cancer Research Fund, UK). The cDNA insert was subcloned into pcDNA3 and 0.45 µg was cotransfected with 0.45 µg pEGFP into L6-GLUT4myc myoblasts. Cells were deprived of serum for 4 h prior to all experimental manipulations. Inhibitors were administered in DMSO and the maximum concentration of the vehicle did not exceed 0.05% (vol/vol). This concentration of the vehicle was without effect effect on any of the parameters measured.

**2-Deoxyglucose uptake.** 2-Deoxyglucose uptake was measured as described (34). After all stimulation and incubations with wortmannin, LY294002 (20 min pretreatment) and insulin (20 min) cell monolayers were washed twice with HEPES-buffered saline (140 mM NaCl, 20 mM HEPES-Na, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM KCl, pH 7.4). Cells were then incubated for 5 min in HEPES-buffered saline containing 10 µM 2-deoxyglucose (1 µCi/ml). Uptake was terminated by washing three times with ice-cold 0.9% NaCl. Non-specific uptake was determined in the presence of 10 µM cytochalasin B and was subtracted from all values. Cell-associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting. Total cellular protein was determined by the Bradford method.

**Detection of cell surface GLUT4myc.** GLUT4myc levels at the cell surface was measured by an antibody-coupled colorimetric assay as described (11). L6-GLUT4myc myotubes were washed once with PBS, fixed with 3% paraformaldehyde (vol/vol) for 3 min at room temperature and then neutralized with 1% glycine (w/vol) in PBS at 4 $^{o}$C for 10 min. Cell monolayers were blocked with 10% goat serum and 3% BSA (w/vol) in PBS at 4 $^{o}$C for at least 30 min. Cells were incubated with anti-c-myc antibody (9E10, 1:100) for 30 min at 4 $^{o}$C. After extensive washing

with PBS cells were incubated for 30 min with peroxidase-conjugated donkey anti-mouse IgG (1:1000, 4 $^{o}$C). Secondary antibody was washed away and 1 ml OPD reagent (0.4 mg/ml o-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate-citrate buffer) was added to each well for 20 min at room temperature. The reaction was stopped by addition of 0.25 ml of 3 M HCl. Optical absorbance of the supernatant was measured at 492 nm.

**Detection of Akt and p38 MAPK phosphorylation**. Lysates were prepared as described previously with modifications (35). Cells were preincubated with wortmannin or LY294002 for 20 min prior to insulin treatment for 10 min in the continued presence of the drugs. Following all appropriate incubations cells were lysed on ice with 150 µM 2X Laemmli sample buffer per well supplemented with 7.5% β-mercaptoethanol (vol/vol), 1 mM $Na_2VO_4$, 100 nM okadaic acid and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 10 µM E-64, 1 µM pepstatin A and 1 µM leupeptin). Lysates were passed 5 times through a 25-gauge syringe and heated for 15 min at 65 $^{o}$C. Fifty micrograms of total protein was resolved by 7.5% or 10% SDS-PAGE to detect phosphorylation of Akt and p38 MAPK, respectively, using phospho-specific antibodies

**Immunoblotting.** Phospho-specific primary antibodies were used at a 1:500 dilution. Anti-p38 MAPK antibody was used at a 1:1000 dilution. Goat anti-rabbit IgG conjugated to horseradish peroxidase was used as secondary antibody at a 1:15 000 dilution. Proteins were detected by the Enhanced Chemiluminescence method according to the manufacturer's instructions (New England Nuclear). Immunoblots were exposed to X-ray film to produce bands within the linear range then quantitated using the National Institutes of Health software, NIH Image.

**Immunoprecipitation and assay of phosphatidylinositol 3-kinase activity in vitro.** PI 3-kinase activity associated with anti-IRS-1 or anti-phosphotyrosine immunoprecipitates was determined as previously described (36) with the following modifications. Three hundred µg of total cellular protein was subjected to immunoprecipitation for 2 h with 2 µg anti-IRS-1 or anti-phosphotyrosine antibodies. Immunoprecipitates were incubated with LY294002 or wortmannin for 5 min prior to initiation of kinase assay and were kept in the presence of the drugs for the duration of the assay. Lipids were separated by thin layer chromatography using Silica gel 60 TLC plates that were pretreated with 1% potassium oxalate. Detection and quantitation of [$^{32}$P]PI3-P on TLC plates was accomplished using a Molecular Dynamics PhosphorImager System (Sunnyvale, CA, USA).

**Immunoprecipitation and assay of Akt and protein kinase C activities.** Immunoprecipitation of Akt isoforms and PKCλ/ζ and in vitro kinase assays were performed as previously described (25) with the following modifications. Cells grown in 6-well plates were treated with wortmannin for 30 min and insulin was added during the final 10 min of this incubation. The cells were lysed and 200 µg of total cellular protein was immunoprecipitated for 2 h with 2 µg of

antibody that was adsorbed to a mixture of protein A- and G-Sepharose beads. Crosstide (150 µM per assay) and myelin basic protein (5 µg per assay) were used as Akt and PKC substrates, respectively. Non-specific activity, determined as activity associated with an irrelevant IgG, was subtracted from all values.

**Immunoprecipitation and assay of p38 MAPK activity.** Protein kinase activity was measured as described (9,19) with modifications Anti-p38α MAPK (rabbit polyclonal) or anti-p38β MAPK (goat polyclonal) antibodies (2 µg per condition) were adsorbed to protein A- or G-Sepharose beads, respectively, by incubating for 2 h at 4 $^{o}$C. Preadsorbed beads were washed twice with 1 ml ice-cold PBS and once with 1 ml ice-cold lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 30 mM sodium pyrophosphate, 10 mM NaF and 1 mM EDTA) supplemented with 1 mM dithiothreitol (DTT), phosphatase inhibitors (1 mM $Na_2VO_4$, and 100 nM okadaic acid) and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 10 µM E-64, 1 µM pepstatin A and 1 µM leupeptin). Lysates were vortexed for 1 min, passed five times through a 25-gauge syringe and centrifuged for 5 min at 12 000 g (4 $^{o}$C). Protein concentration of the supernatant was determined by the bicinchoninic acid method according to the manufacturer's instruction (Pierce, Rockford, IL). p38 MAPK was immunoprecipitated by incubating 250 µg of total protein for 2-3 h with the pre-adsorbed Sepharose beads. Immunocomplexes were isolated and washed four times with 1 ml wash buffer (25 mM HEPES, pH 7.8, 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 0.1% bovine serum albumin (w/vol) and 1 M NaCl) supplemented with 1 mM DTT, 1 mM PMSF and phosphatase inhibitors (1 mM $Na_2VO_4$, and 10 nM okadaic acid) and twice with 1 ml kinase buffer (50 mM Tris/HCl, pH 7.5 and 10 mM $MgCl_2$ supplemented with 1 mM $Na_2VO_4$, and 10 nM okadaic acid.

Immunocomplexes were then incubated for 30 min at 30 $^{o}$C with 30 µl reaction mixture (kinase buffer containing 2 µg ATF2, 150 µM ATP and 2 µCi [$\gamma^{32}$P]ATP per condition) on a platform shaker. Reaction was stopped by addition of 30 µl 2X Laemmli sample buffer and heating for 30 min at 37 $^{o}$C. Samples were centrifuged for 5 min (12 000 g) then 40 µl of the supernatant was resolved by 13% SDS-PAGE and electrotransferred onto PVDF membranes. The amount of radiolabeled phosphate transferred onto the substrate was determined by exposing the PVDF membrane to a PhosphorImager cassette and quantitated using a Molecular Dynamics PhosphorImager System (Sunnyvale, CA, USA). Equal protein loading was confirmed by immunoblotting the PVDF for the respective p38 MAPK isoform following quantitation.

**Indirect immunofluorescence and measurement of GLUT4myc translocation and p38 MAPK phosphorylation.** Phosphorylation of p38 MAPK in single cells was detected by indirect immunofluorescence using a monoclonal phospho-specific antibody which recognizes p38 MAPK when phosphorylated on Y182(1:200 dilution). Indirect immunofluorescence was measured as described previously (25). GLUT4myc at the cell surface was detected in unpermeablized cells using a monoclonal anti-myc antibody (9E10, 2 µg/ml). Transfected cells were identified by expression of green fluorescent protein. Secondary antibody (Cy3-conjugated goat anti-mouse) was used in a 1:1000 dilution.

## Results

### Glucose uptake has a higher sensitivity to wortmannin than GLUT4 translocation

We first determined if there was a differential sensitivity of glucose uptake and GLUT4 translocation to wortmannin in muscle cells in culture. To accurately and quantitatively detect GLUT4 molecules that are fully inserted into the plasma membrane of intact cells, we used L6 muscle cells stably expressing GLUT4 tagged with an exofacial myc epitope (L6-GLUT4myc cells). By incubating the monolayer of intact cells with an anti-myc antibody, we were able to quantitate the change in cell surface GLUT4 without the need for subcellular fractionation. L6-GLUT4myc myotubes were pretreated with the indicated concentrations of wortmannin for 20 min, prior to insulin stimulation for an additional 20 min, in the continued presence of the drug. The relative amount of GLUT4myc at the cell surface and 2-deoxyglucose uptake are shown in Fig. 1A. Insulin increased 2-deoxyglucose uptake by 2.3-fold (basal: 8.2 ± 0.4, insulin: 18.6 ± 1.2 pmol/min/mg protein) and GLUT4myc at the cell surface by 2.4 ± 0.1-fold. Insulin-stimulated 2-deoxyglucose uptake or GLUT4myc translocation in the absence of wortmannin is expressed as 100% in Fig 1A. Wortmannin treatment abolished the stimulation of glucose uptake by insulin with an $IC_{50}$ of 3 nM. In contrast to the stimulation of glucose uptake, the gain in GLUT4myc at the plasma membrane was less sensitive to inhibition by wortmannin ($IC_{50}$ = 43, Fig. 1A). Translocation of GLUT4 was unaffected by concentrations of wortmannin which reduced glucose uptake by more than 60%. These results suggest that GLUT4 activity and GLUT4 translocation are regulated by signaling pathways with high and low sensitivities to wortmannin, respectively.

### LY294002 inhibits glucose uptake and GLUT4 translocation with similar potency

LY294002 is a PI 3-kinase inhibitor that acts by binding within the ATP binding pocket of the enzyme (37). We reasoned that if the translocation and activation of GLUT4 are mediated by PI 3-kinases with different affinities for wortmannin, then these two phenomena might also display different sensitivities to LY294002. Cell surface GLUT4myc levels and 2-deoxyglucose uptake were determined in cells that were treated for 20 min with LY294002 prior to insulin treatment. The results illustrated in Fig. 1B demonstrate that both insulin-stimulated glucose uptake and GLUT4 translocation were inhibited by LY294002 with superimposable dose-dependencies. The calculated $IC_{50}$ values for inhibition of glucose uptake and GLUT4 translocation by LY294002 were 8 and 10.5 μM, respectively (see table 1). Interestingly, 50 μM LY294002 inhibited GLUT4 translocation completely but reduced glucose uptake by only 70%, whereas wortmannin fully inhibited both insulin responses.

**Sensitivity of PI 3-kinase to wortmannin and LY294002**

Given the different effects of wortmannin and LY294002 described above, we determined the sensitivity of PI 3-kinase to these two inhibitors in an in vitro assay. Insulin-receptor substrate (IRS)-1 or phosphotyrosine-containing proteins were immunoprecipitated from cells that were treated for 10 min with insulin. PI 3-kinase activity associated with these immunoprecipitates was measured in the presence of wortmannin or LY294002 and the results are illustrated in Fig. 2. Insulin increased IRS-1-associated PI 3-kinase activity by 13 ± 2-fold. This PI 3-kinase activity was inhibited in vitro by wortmannin (Fig. 2A) and LY294002 (Fig. 2C) with $IC_{50}$ values of 0.3 nM and 0.9 μM, respectively (table 1). PI 3-kinase activity associated with anti-phospho-tyrosine immunoprecipitates was increased 24-fold ± 3-fold by insulin

treatment. This activity was also inhibited in vitro by wortmannin (Fig. 2B) and LY294002 (Fig. 2D) with $IC_{50}$ values of 0.5 nM and 0.7 μM, respectively (table 1).

**Inhibition of insulin-induced Akt phosphorylation and kinase activity by wortmannin and LY294002 correlates with GLUT4 translocation but not glucose uptake**

We next performed a detailed analysis of the effect of wortmannin and LY294002 on the signals leading to GLUT4 translocation. In particular, we looked at the effect of wortmannin and LY294002 on the phosphorylation and activation of Akt in intact cells. Phosphorylation of Akt on two residues (T308 and S473) is required for its full activation (38). The phosphorylation status of the enzyme can be monitored using phospho-specific antibodies, directed to either of these two sites. These antibodies recognize the three isoforms of Akt (Akt1, 2 and 3) expressed in L6 cells (Sweeney, G., J. Keen, Somwar, R. and A. Klip, unpublished observation). Cell lysates prepared from cells treated with insulin and either wortmannin or LY294002 were immunoblotted for Akt phosphorylated at either T308 or S473. Insulin-stimulated Akt phosphorylation in the absence of any inhibitor is expressed as 100% in Figs. 3 and 4. As shown, wortmannin reduced insulin-stimulated T308 and S473 phosphorylation of Akt with $IC_{50}$ of 29 and 25 nM, respectively (Figs. 3A and B and table 1). LY294002 repressed phosphorylation of Akt at T308 and S473 with $IC_{50}$ values of 14 and 18 μM, respectively (Fig. 4 and table 1).

Although phosphorylation of Akt correlates with its activity (38), direct measurement of kinase activity of the three isoforms expressed in these cells (39) provides a more accurate reflection of enzyme regulation. Akt1, 2 and 3 were immunoisolated from cells that had been treated with wortmannin and/or insulin and enzyme activity measured by an in vitro kinase assay (Fig. 3C). Enzyme activity of each isoform observed in the presence of insulin alone is expressed

as 100%. Activation of the three enzymes by insulin was prevented by wortmannin with $IC_{50}$ of 30 nM (Akt1), 35 nM (Akt2) and 60 nM (Akt3). Hence, the ability of wortmannin to inhibit insulin-induced Akt activation correlates more closely with the reduction in GLUT4 translocation than in glucose uptake (Fig. 1A and table 1).

**Inhibition of insulin-stimulated atypical PKC activity by wortmannin correlates with inhibition of GLUT4 translocation but not glucose uptake**

Atypical PKC isoforms are also believed to participate in the stimulation of GLUT4 translocation (30). PKCλ/ζ was immunoprecipitated with an antibody (C-20) which recognizes both isoforms, from cells that were treated with wortmannin and/or insulin. Enzyme activity was measured by an in vitro kinase assay. Insulin enhanced PKCλ/ζ activity by 3.2 ± 0.3-fold. This activity is expressed as 100% in Fig. 5. Wortmannin pretreatment reduced insulin-stimulated PKCλ/ζ activation with an $IC_{50}$ of 24 nM (table 1). As was the case with Akt, this sensitivity closely parallels that of insulin-stimulated GLUT4 translocation.

**Activation of p38 MAPK by insulin is prevented by wortmannin with a potency that parallels the inhibition of glucose uptake**

The results illustrated in Fig. 1 suggested that regulation of GLUT4 activity by insulin occurred via a wortmannin-sensitive mechanism. We have reported that GLUT4 activity is reduced by pharmacological inhibitors of p38 MAPK (9,13,14). These observations led us to hypothesize that activation of p38 MAPK by insulin may involve a wortmannin-sensitive target. To test this possibility we determined the effect of wortmannin on p38 MAPK phosphorylation and kinase activity. Phosphorylation of the enzyme on T180 and Y182 by upstream kinase(s) is

indispensable for activation (40). We detected p38 MAPK phosphorylation by immunoblotting with an antibody that recognizes the dual phosphorylated enzyme (41). Insulin increased p38 MAPK phosphorylation by 2.4-fold and this is expressed as 100% in Fig. 6A. Pretreatment of myotubes with wortmannin reduced insulin-stimulated p38 MAPK phosphorylation with $IC_{50}$ of 6 nM (table 1). This effect was specific for insulin as phosphorylation of p38 MAPK elicited by anisomycin or mannitol was not affected by wortmannin treatment (data not shown).

The phospho-specific antibody used here does not discriminate between p38 MAPK isoforms. Therefore, we also determined the effect of wortmannin on the activation of individual p38 MAPK isoforms. We have previously shown that SB203580 which inhibits only p38α and p38β MAPK (42), reduced insulin-stimulated glucose uptake (9). Hence, we focused on the effect of wortmannin on these isoforms. p38α MAPK activity was increased by 2.1 ± 0.1-fold when cells were incubated with insulin. Similar to glucose uptake and p38 MAPK phosphorylation, this activity was highly sensitive to inhibition by wortmannin treatment of cells (Fig. 6B). The $IC_{50}$ calculated for this effect was 6 nM (table 1). We also immunoprecipitated active p38 MAPK using an immobilized phospho-specific p38 MAPK antibody from cells that were pretreated with 10 or 100 nM wortmannin, prior to insulin treatment for 10 min. These immunoprecipitates were then immunoblotted for p38α MAPK. The amount of p38α MAPK that could be detected in anti-phospho p38 MAPK immunoprecipitates in insulin treated cells was decreased by approximately 80% when myotubes were pretreated with 10 nM wortmannin and completely inhibited by 100 nM wortmannin (insulin: 3.2-fold above basal, 10 nM wortmannin + insulin: 1.3-fold above basal, 100 nM + insulin: 0.5-fold of basal, n = 1).

As observed for p38α MAPK, insulin increased p38β MAPK activity by 2.6 ± 0.2-fold. Activation of p38β MAPK was also very sensitive to inhibition by pretreatment of cells with

wortmannin (Fig. 6C). The $IC_{50}$ calculated for this effect was 2 nM (table 1). A strong correlation between wortmannin-mediated inhibition of p38 MAPK activation and reduction of glucose uptake, but not GLUT4 translocation, is evident when plotting these parameters together (Fig. 6D). For example, 90% of insulin-stimulated p38 MAPK phosphorylation was inhibited by 25 nM wortmannin while glucose uptake was reduced by approximately 80% under the same conditions. At this concentration of wortmannin, insulin-induced GLUT4 translocation was reduced by only 25%.

**Insulin-stimulated phosphorylation of p38 MAPK is resistant to inhibition by LY294002**

The increase in p38 MAPK phosphorylation in response to insulin (2.4-fold) was resistant to inhibition by LY294002 (Fig. 7A). A significant reduction in insulin-induced p38 MAPK phosphorylation was only observed with 50 μM LY294002 (58.8 ± 10.7%). No further reduction was observed at higher concentrations (up to 100 μM, data not shown). Consistent with the results illustrated in Fig. 1B, these findings indicate that the signal pathway regulating the activation of GLUT4 by insulin (unveiled by its high sensitivity to wortmannin and SB203580) is not sensitive to inhibition by LY294002.

**A dominant negative mutant of the regulatory p85 subunit of PI 3-kinase inhibits insulin-stimulated GLUT4 translocation but not p38 MAPK phosphorylation**

The results discussed above indicate that insulin increases p38 MAPK activity by a wortmannin-sensitive but LY294002-insensitive pathway. To further explore the role of PI 3-kinases in the activation of p38 MAPK, we determined the effect of transient expression of a mutant PI 3-kinase (Δp85) which acts dominantly to inhibit activation of Type IA PI 3-kinases.

We monitored insulin-stimulated p38 MAPK phosphorylation by immunofluorescence in single cells as described previously (43). Transfected cells are indicated by arrows and were identified by co-expression of green fluorescent protein (GFP, shown in lower panels of Fig. 8). Similar to results in Figs. 5 and 6, p38 MAPK phosphorylation was increased by insulin in untransfected cells (Fig. 8A). Expression of Δp85 had no effect on insulin-stimulated phosphorylation of p38 MAPK (Fig. 8B, top left), compared to the surrounding untransfected cells. Immunofluorescent detection of cell surface GLUT4myc was also measured by labeling intact cells with anti-myc antibody. As expected, GLUT4myc gain at the cell surface in response to insulin was abrogated by expression of Δp85 (Fig. 8B, top right). These results indicate that activation of p38 MAPK is not dependent on a p85/p110 type of PI 3-kinase.

## Discussion

Several studies have shown that increasing the amount of GLUT4 at the cell surface of skeletal muscle (2), rat adipocytes (7), L6 muscle cells (9,13) and 3T3-L1 adipocytes (9,10) is not sufficient to elicit maximum stimulation of glucose uptake. In addition, it has been demonstrated that GLUT4 translocation precedes the stimulation of glucose uptake in L6 muscle cells (13) and rat adipocytes (7). A view that emerged from these studies is that two events culminate in full stimulation of glucose uptake: a) translocation of GLUT4 to the cell surface, and b) increase in GLUT4 activity.

### Differential sensitivity of glucose uptake and GLUT4 translocation to wortmannin

The differential sensitivity to wortmannin of the stimulation of glucose uptake and GLUT4 translocation by insulin was the first evidence for a dual input resulting in glucose uptake in this study. Glucose uptake was inhibited by wortmannin with an $IC_{50}$ of 3 nM. In contrast, the drug prevented the arrival of GLUT4 at the cell surface with an $IC_{50}$ of 43 nM. The assay used to determine the amount of GLUT4 on the cell surface is based on quantitative immunological detection of the exofacial myc epitope on the GLUT4 molecule, i.e., it detects transporters that are fully inserted in the plasma membrane and exposed to the extracellular milieu. Glucose transport therefore, displayed a higher sensitivity to wortmannin than GLUT4 translocation, by one order of magnitude. A similar observation was made in 3T3-L1 adipocytes (10) where the $IC_{50}$ of the drug was 6 nM for glucose uptake and 80 nM for GLUT4 translocation. In the latter study, GLUT4 translocation was detected by exposure of another exofacial epitope using fluorescence microscopy (10). Collectively, these results suggest that two different wortmannin targets might regulate these processes. It is unlikely that the inhibition of

glucose uptake by wortmannin is due to a direct interaction of wortmannin with GLUT4, since the drug had no effect on the stimulation of glucose uptake by dinitrophenol in L6 myotubes (44) or by contraction of rat skeletal muscle (20,45) at concentrations as high as 1 μM. In addition, wortmannin does not affect the stimulation of glucose uptake caused by expression of constitutively active Akt mutants in L6 myotubes and 3T3-L1 adipocytes (10,46,47). It is easy to envisage that concentrations of wortmannin that inhibit GLUT4 translocation (> 25 nM) will prevent stimulation of glucose uptake in response to insulin. However, the fact that 60% of the insulin response of glucose uptake is inhibited at concentrations that do not affect GLUT4 availability at the cell surface, and that wortmannin does not affect GLUT4 per se, suggest the existence of a different signal targeted by the drug that leads to activation of the translocated transporters.

To begin to examine whether the differential sensitivity to wortmannin of GLUT4 translocation and putative activation of GLUT4 is due to different PI 3-kinase inputs (e.g., through different PI 3-kinase isoforms or PI 3-kinase products), we compared the sensitivity of both phenomena to another chemically unrelated inhibitor of PI 3-kinase, LY294002. Interestingly, glucose uptake and GLUT4 translocation were inhibited with similar potency by LY294002. The simplest interpretation is that the reduction in glucose uptake observed in the presence of LY294002 is due mainly to a reduction in cell surface GLUT4 levels. Intriguingly, LY294002 reduced the stimulation of glucose uptake by only 70% when GLUT4 translocation was fully blocked. In contrast, in the presence of wortmannin, the stimulation of glucose uptake was completely abrogated when cell surface GLUT4 was reduced to basal levels. These observations raise the possibility that GLUT4 activity may be regulated by either a PI 3-kinase

that is highly sensitive to wortmannin but not to LY294002 or by another wortmannin-sensitive target which is not a PI 3-kinase.

When the sensitivity to wortmannin was measured in vitro, PI 3-kinases associated with IRS-1 or phosphotyrosine-containing proteins were inhibited with $IC_{50}$ values of 0.3 and 0.5 nM, respectively. These values are clearly lower than even those determined for inhibition of glucose uptake in intact cells, consistent with the view that, in vivo, higher concentrations of wortmannin may be needed to inhibit PI 3-kinases. It has recently been suggested that this difference may be due to the high concentration of intracellular ATP that competes with wortmannin for binding to PI 3-kinase (48). We were unable to determine the $IC_{50}$ for inhibition of PI 3-kinase by wortmannin in intact cells because the drug does not remain bound to the enzyme following isolation of the IRS-1 or phosphotyrosine immunoprecipitates. Therefore, it is difficult to draw any conclusion about the participation of PI 3-kinases in GLUT4 activation or GLUT4 traffic based solely on the sensitivity to wortmannin of PI 3-kinase measured in vitro.

**Inhibition of the signaling pathway regulating GLUT4 activation and the stimulation of glucose uptake by wortmannin but not LY294002 or Δp85**

We recently demonstrated that several structurally different inhibitors of p38 MAPK reduced glucose uptake but not GLUT4 translocation (9,13) in both L6 myotubes and 3T3-L1 adipocytes. This situation is highly reminiscent of the observations reported in the present study at low concentrations of wortmannin. In addition, in our hands expression of a dominant negative p38 MAPK mutant in 3T3-L1 adipocytes reduced glucose uptake (49). Together, these studies suggested that GLUT4 activity may be regulated by a p38 MAPK-dependent pathway. Accordingly, we report here that the stimulation of p38 MAPK activity by insulin also displayed

high sensitivity to inhibition by wortmannin. Insulin-stimulated p38 MAPK phosphorylation and activation of p38α and p38β isoforms were inhibited by wortmannin with $IC_{50}$ values of 7, 6 and 2 nM, respectively. It is unlikely that wortmannin interacts directly with p38 MAPK since wortmannin prevented insulin-induced phosphorylation of p38 MAPK, a step that is dependent on an upstream kinase. In addition, treatment of intact cells with wortmannin had no effect on the activation of p38 MAPK by anisomycin or mannitol (data not shown). Furthermore, it was recently reported that wortmannin did not inhibit recombinant p38 MAPK in in vitro kinase assays (48). Our results support the view that insulin engages a wortmannin-sensitive target to activate p38 MAPK leading to enhanced GLUT4 activity and maximum stimulation of glucose uptake. The identity of this high-affinity wortmannin target remains to be determined.

Contrary to the exquisite sensitivity of the activation of p38 MAPK by insulin to wortmannin, this response was largely unaffected by concentrations of LY294002. Only concentrations of LY294002 higher than those required for inhibition of GLUT4 translocation affected p38 MAPK phosphorylation. This result is consistent with the observation, described above, that there was no detectable reduction in GLUT4 activity by LY294002 independently of GLUT4 translocation. Moreover, expression of Δp85 to inhibit Type IA PI 3-kinases, did not reduce p38 MAPK phosphorylation, akin to the results obtained with LY294002. Under similar conditions, Δp85 completely prevented the arrival of GLUT4 at the cell surface in response to insulin. These results support the notion that insulin activates p38 MAPK through a mechanism that involves a highly sensitive wortmannin target that is not a Type IA PI 3-kinase. In agreement with this conclusion, it was reported that wortmannin inhibits bombesin-stimulated cytosolic phospholipase $A_2$ ($cPLA_2$) activity in Swiss 3T3 cells with an $IC_{50}$ of 2 nM (50). This inhibition of enzyme activity was not due to inhibition of PI 3-kinase and could not be accounted

for by direct inhibition of $cPLA_2$ by wortmannin (50). These studies support the existence of a highly sensitive wortmannin target.

**Signaling pathway regulating GLUT4 translocation**

In contrast to the emerging knowledge about the signals regulating GLUT4 activity, much more is known about the signals that meditate insulin-stimulated GLUT4 translocation. Many studies have shown that PI 3-kinase plays an important role in mediating this effect. The numerous experimental approaches showing a need for PI 3-kinase include the use of the pharmacological inhibitors wortmannin (22,51) and LY294002 (23) and expression of both inhibitory (25,52) and constitutively active (21,53) mutant constructs of PI 3-kinase. More recently, microinjection of an antibody to the p110 catalytic subunit of PI 3-kinase (10) or of peptides encompassing the SH2 domain of p85 (54) resulted in a reduction in insulin-stimulated GLUT4 translocation. Microinjection of antibody to the 3' lipid phosphatase PTEN increased basal and insulin-stimulated GLUT4 translocation while overexpression of PTEN reduced basal and insulin-stimulated GLUT4 translocation and glucose uptake (55). We have shown that GLUT4 translocation (56), but not glucose uptake (56,57), is stimulated by the introduction of phosphatidylinositol 3,4,5-$P_3$ ($PIP_3$) into 3T3-L1 adipocytes and L6 muscle cells (57). This finding supports the notion that at least this PI 3-kinase product may not be involved in regulating GLUT4 activity. Alternatively, it is possible that GLUT4 translocation requires a lower level of $PIP_3$ than is required for the stimulation of glucose uptake. Such a scenario would explain why a higher concentration of wortmannin is needed to fully inhibit GLUT4 translocation. However, this remains a weak possibility because we did not observe a differential sensitivity to LY294002 of glucose uptake and GLUT4 translocation, in agreement with results

in 3T3-L1 adipocytes (10). Strengthening this argument would require measuring the endogenous levels of PI 3-kinase products under the different conditions and the subcellular location of these lipids. PI 3-kinase products are thought to mediate GLUT4 translocation by activating Akt and/or atypical PKC isoforms (29,58). In the present study, Akt isoforms and PKCζ/λ activities were inhibited by wortmannin with $IC_{50}$ values that were similar and correlated closely with inhibition of GLUT4 translocation.

In summary, our results suggest a segregation of signaling events leading to GLUT4 translocation and GLUT4 activation. We demonstrate here a differential sensitivity of insulin-stimulated GLUT4 translocation and glucose uptake to wortmannin. The lower sensitivity of GLUT4 translocation correlates with the sensitivity of insulin-induced Akt and PKCζ/λ activity to inhibition by wortmannin. In contrast, the high sensitivity of glucose uptake to inhibition by wortmannin correlates with inhibition of p38 MAPK. Collectively, these results suggest that a cellular target of insulin with a high affinity for wortmannin regulate the activation of p38 MAPK and GLUT4. We also suggest that such target is unlikely to be a Type IA PI 3-kinase since LY294002 and Δp85 did not inhibit insulin-stimulated p38 MAPK phosphorylation.

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## Footnotes

[1] GLUTs: glucose transporters
MAPK: mitogen-activated protein kinase
PI: PI 3-kinase
PKB:protein kinase B
PKC: protein kinase C
ATF2: activating transcription factor 2
GFP: green fluorescent protein
PBS: phosphate buffered saline
BSA: bovine serum albumin
OPD: O-phenylenediamine dihydrochloride

## Figure Legends

**Figure 1. Sensitivity of glucose transport and GLUT4 translocation to wortmannin and LY294002.** L6-GLUT4myc myotubes were left untreated or treated for 20 min with the indicated concentrations of wortmannin (A) or LY294002 (B) prior to simulation with insulin for 20 min, in the absence or continued presence of inhibitors. Cell surface GLUT4myc or 2-deoxyglucose uptake were then determined as described in Materials and Methods. Results are the mean ± SE of 4 experiments in which each condition was assayed in triplicate determinations. Insulin-dependent 2-deoxyglucose uptake or GLUT4myc translocation in the absence of inhibitor were considered as 100%.

**Figure 2. In vitro sensitivity of PI 3-kinase to wortmannin and LY294002.** Insulin receptor substrate-1 (IRS-1, A and C) or phosphotyrosine (PY)-containing proteins (B and D) were immunoprecipitated from control or insulin-treated (5 min) cells. PI 3-kinase activity associated with each immunoprecipitate, towards PI, was determined in the presence of the indicated concentrations of wortmannin (A and B) or LY294002 (C and D) in vitro. Results are the mean ± SE of 3 to 4 experiments in which each condition was assayed in duplicate.

**Figure 3. Sensitivity of Akt phosphorylation and kinase activity to wortmannin.** Cells were treated with the indicated concentrations of wortmannin (20 min) prior to insulin treatment for 10 min in the continued presence of wortmannin. Whole cell lysates (50 µg) were immunoblotted to detect Akt phosphorylated on T308 (A) or S473 (B). Representative immunoblots are shown. Immunoblots were scanned within the linear range, quantitated and the results of 4 to 5 experiments are illustrated in the graph below each immunoblot. Protein kinase activity of the

different Akt isoforms was determined using an in vitro kinase assay (C) as described in Materials and Methods. Results are the mean ± SE of 4 experiments. Insulin-stimulated phosphorylation or kinase activity in the absence of wortmannin was considered as 100%.

**Figure 4. Inhibition of Akt phosphorylation by LY294002.** Lysates were prepared from cells that were treated for 20 min with the indicated concentration of LY294002 prior to stimulation with insulin for an additional 10 min. Phosphorylated Akt was detected and quantitated as described in the legend to figure 3.

**Figure 5. Inhibition of PKCζ/λ activity by wortmannin.** PKCζ/λ was immunoisolated from cell extracts prepared from cells that were treated with the indicated concentration of wortmannin prior to insulin treatment for an additional 10 min. Kinase activity was determined by an in vitro kinase assay as described in Materials and Methods. Insulin-stimulated kinase activity in the absence of wortmannin was considered as 100%. Results are the mean ± SE of 3 experiments.

**Figure 6. Inhibition of p38 MAPK phosphorylation and activity by wortmannin.** Lysates prepared from cells that were treated as described in the legend to figure 3 were immunoblotted to detect phosphorylated p38 MAPK (A). A representative immunoblot is shown. The results of 4 immunoblots, scanned within the linear range, were quantitated and are illustrated in the graph below. (B) Protein kinase activity of p38 MAPK alpha or beta isoforms was determined by an in vitro kinase assay. Results are the mean ± SE of 4 experiments. Insulin-stimulated

phosphorylation or kinase activity in the absence of wortmannin was considered as 100%. All values are expressed relative to this.

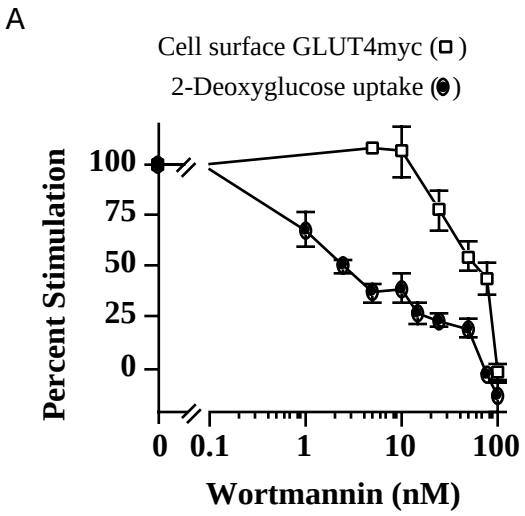
**Figure 7. p38 MAPK phosphorylation is resistant to inhibition by LY294002.** Lysates were prepared from cells that were treated for 20 min with the indicated concentration of LY294002, prior to insulin treatment for 10 min. Detection and quantitation of p38 MAPK phosphorylation was done as described in the legend to figure 3. Insulin-stimulated phosphorylation in the absence of LY294002, was considered as 100%. Results are the mean ± SE of 4 experiments.

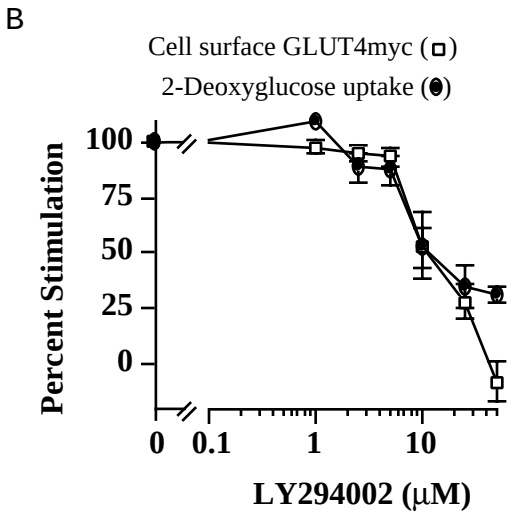
**Figure 8. Dominant negative PI 3-kinase inhibits GLUT4 translocation but not p38 MAPK phosphorylation.** (A) Untransfected cells were left untreated or treated with insulin for 10 min. Phosphorylation of p38 MAPK was detected by immunofluoresence in permeabilized cells, using a phospho-specific p38 MAPK antibody as described in Materials and Methods. (B) L6 cells were transiently transfected with the cDNAs (0.4 μg of each) for Δp85 and green fluorescent protein (GFP) and then incubated for 10 min with insulin. Phosphorylated p38 MAPK (permeabilized cells, left panels) or cell surface GLUT4 (unpermeablized cells, right panels) were then detected by immunofluoresence. Arrows in the upper panels indicate transfected cells, identified by expression of GFP in the lower panels. Similar results were obtained in 3 experiments.

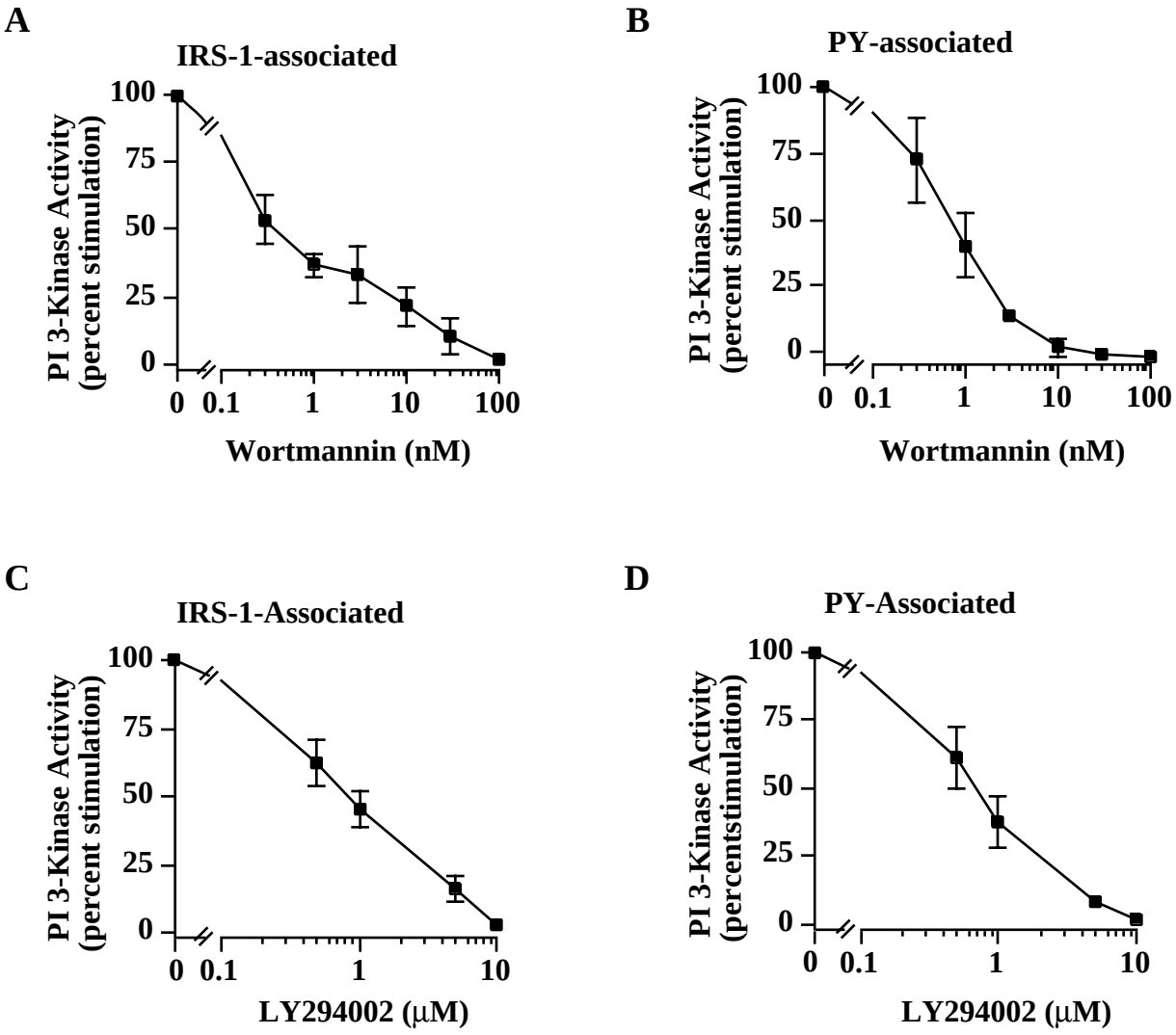
| | Wortmannin (nM) | LY294002 (μM) |
|---|---|---|
| **2-Deoxyglucose uptake** | 3 | 8 |
| **GLUT4myc translocation** | 43 | 10 |
| **PI 3-kinase activity** | | |
| IRS-1-associated | 0.3 (in vitro) | 0.9 (in vitro) |
| PY-associated | 0.5 (in vitro) | 0.7 (in vitro) |
| **Akt** | | |
| Akt phosphorylation (T308) | 29 | 14 |
| Akt phosphorylation (S473) | 25 | 18 |
| Akt1 kinase activity | 35 | ND |
| Akt2 kinase activity | 30 | ND |
| Akt3 kinase activity | 60 | ND |
| PKCζ/λ kinase activity | 24 | ND |
| **p38 MAPK** | | |
| p38 MAPK phosphorylation | 7 | Small reduction, only at >25 μM |
| p38α MAPK activity | 6 | ND |
| p38β MAPK activity | 2 | ND |

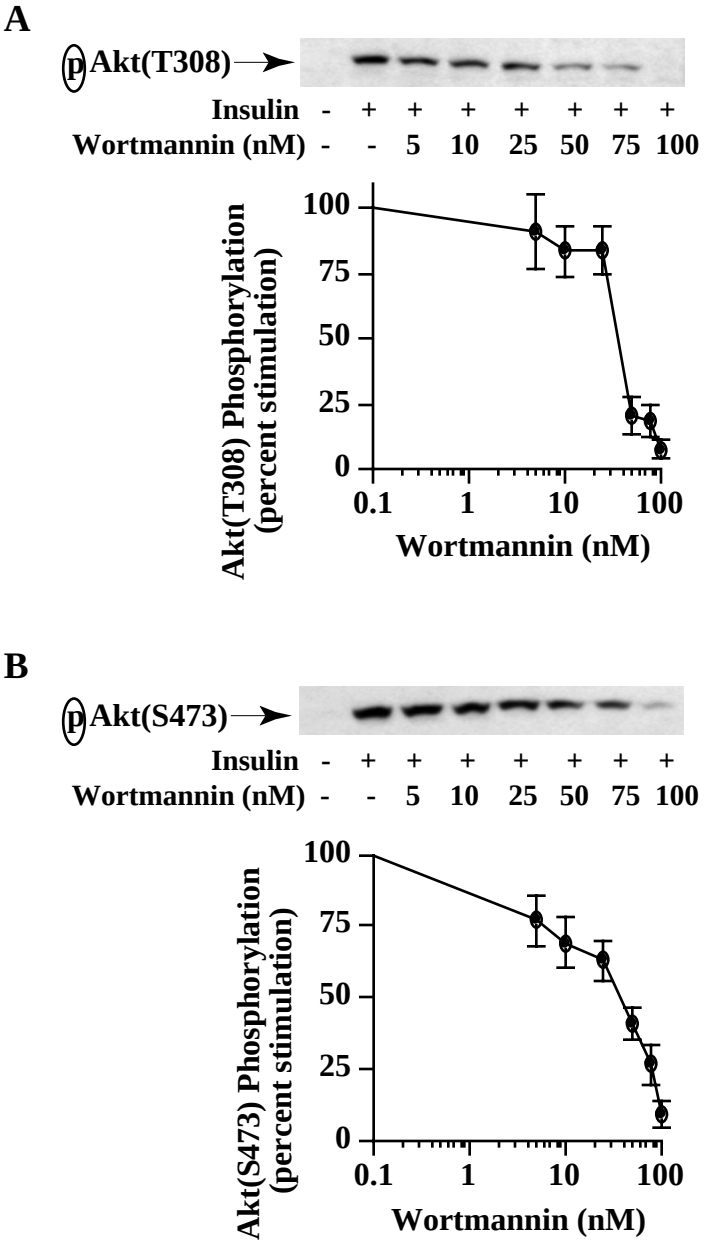
**Table 1. $IC_{50}$ values for the inhibition of insulin-stimulated glucose uptake, GLUT4myc translocation and kinase activities by wortmannin and LY294002.** These values were calculated from the data presented in text and in figures 1 to 7. Except for the assay of PI 3-kinase activity, wortmannin and LY294002 were given to intact cells for 20 min prior to insulin

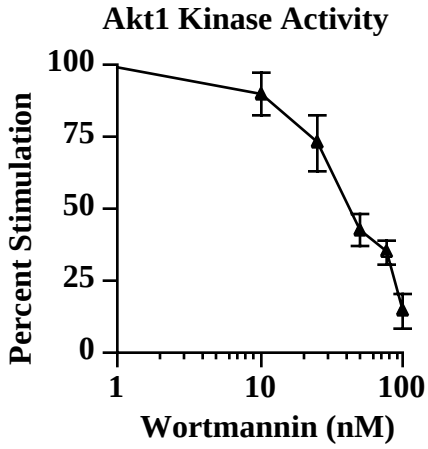
and remained present throughout the insulin treatment. PY: phosphotyrosine, IRS-1: insulin receptor substrate-1, ND: not determined.

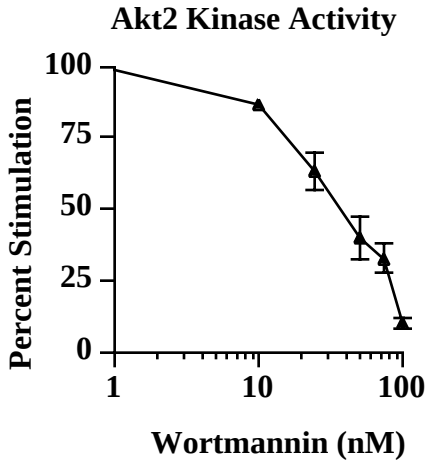


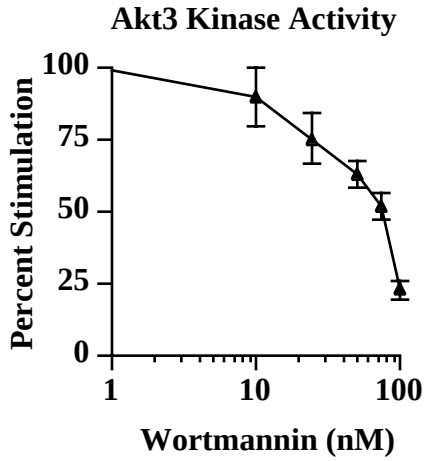



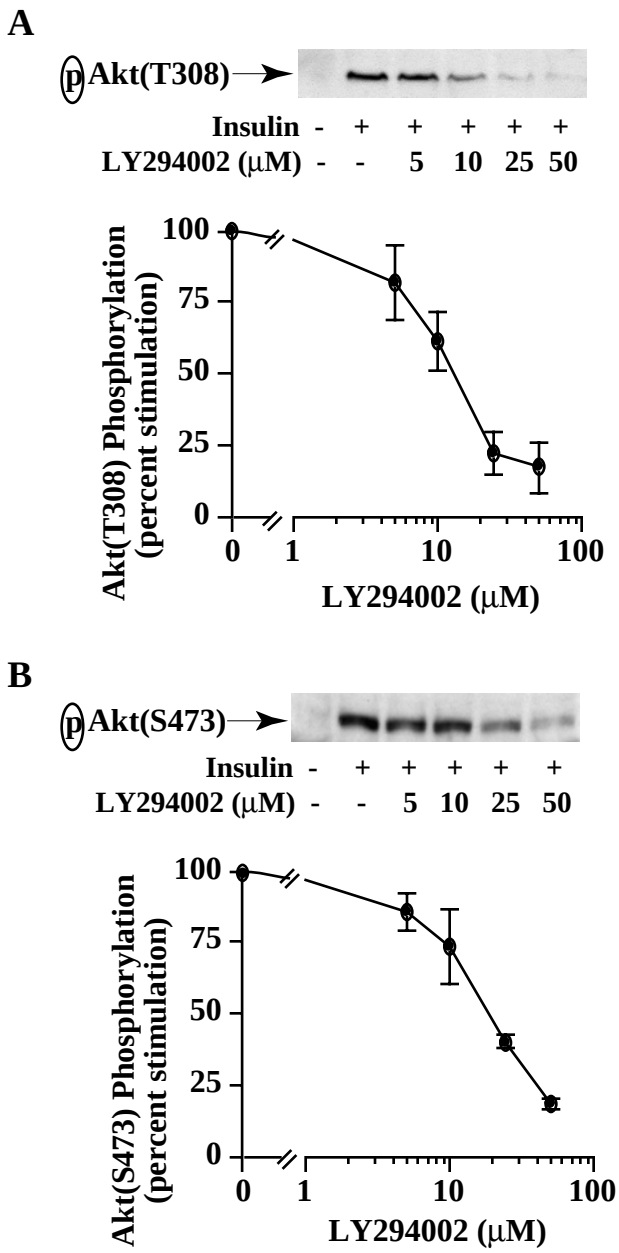


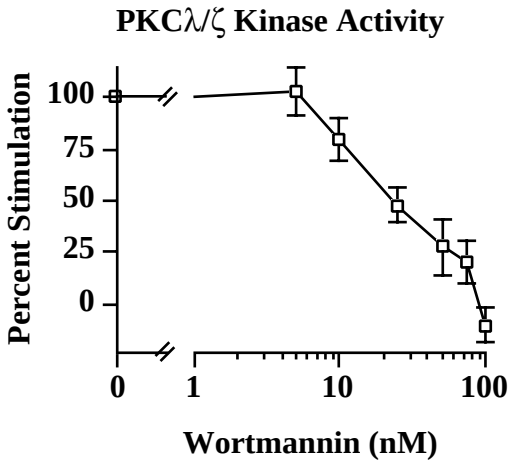


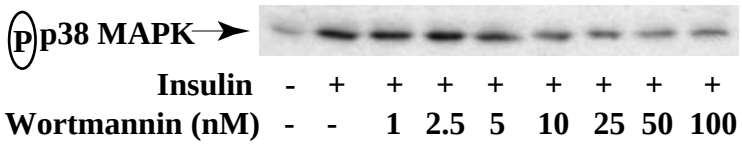


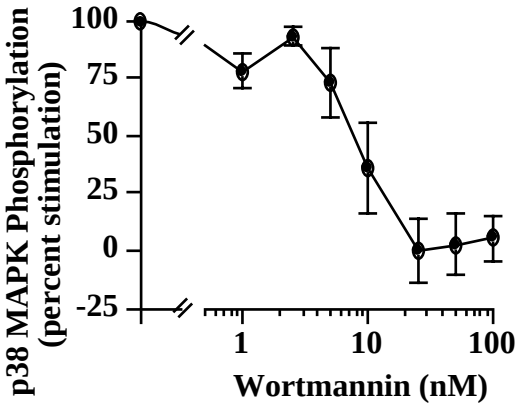



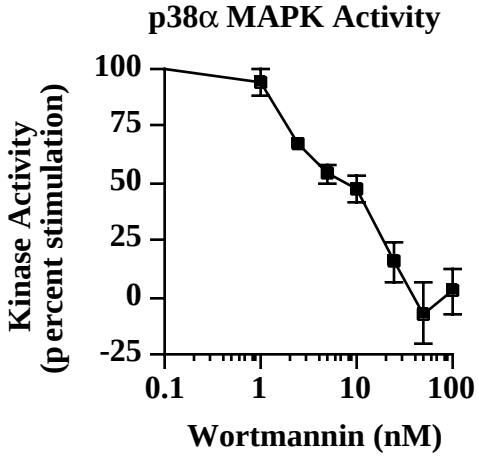


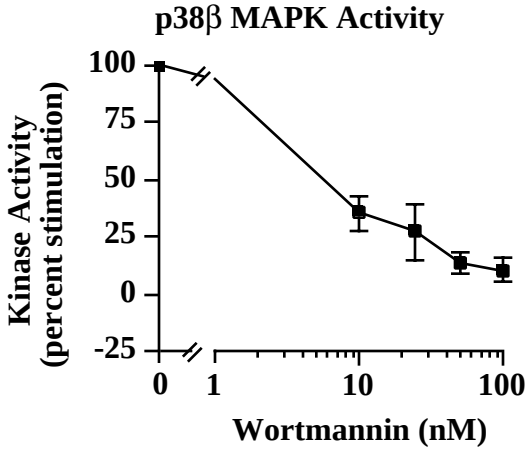


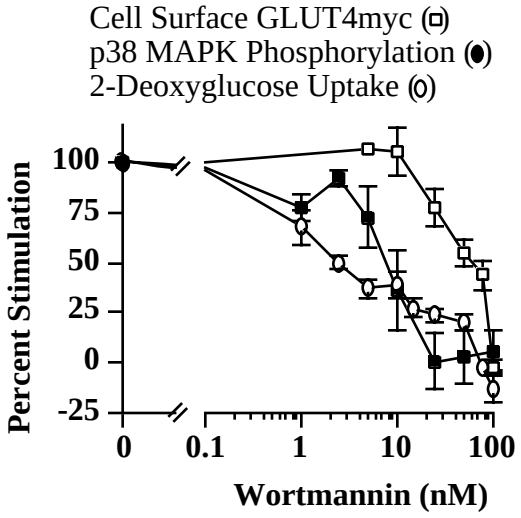



C



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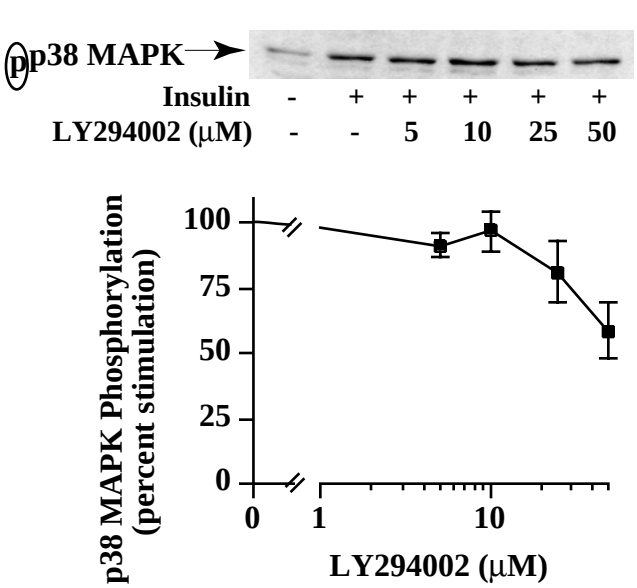


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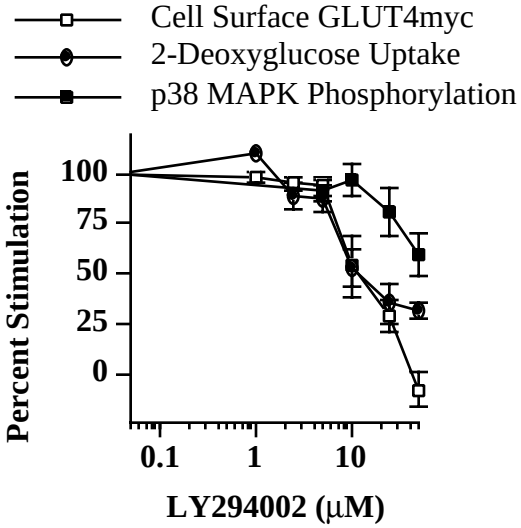

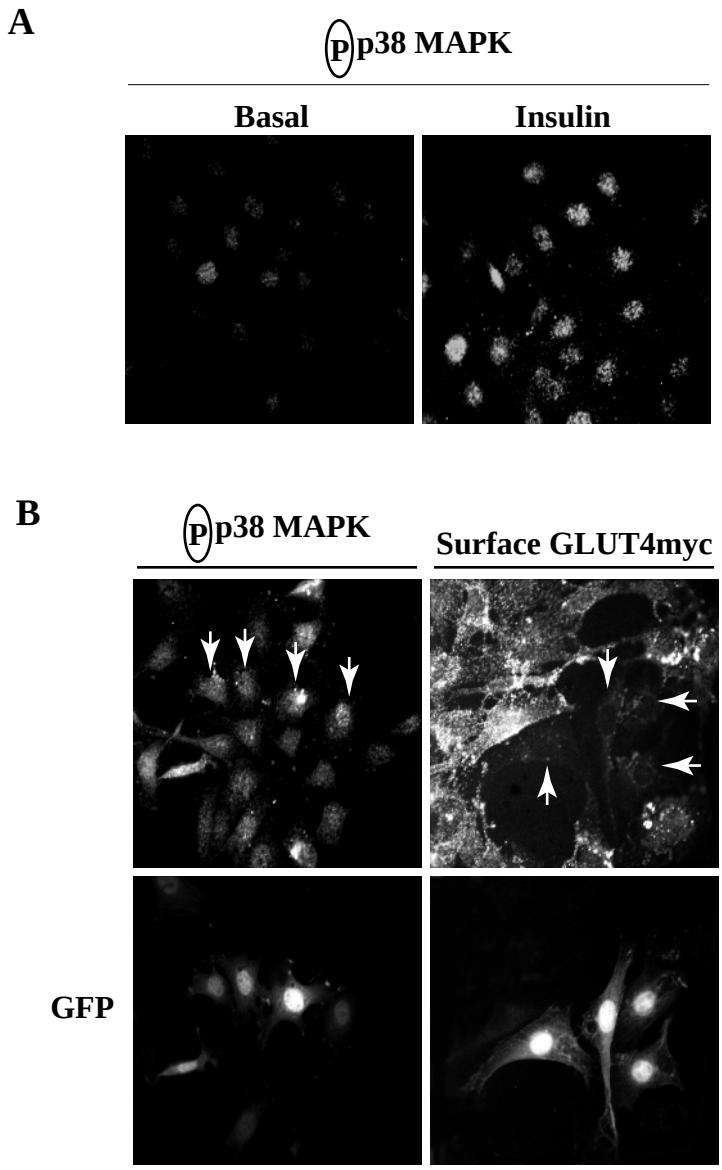

**Differential effects of phosphatidylinositol 3-kinase inhibition on intracellular signals regulating GLUT4 translocation and glucose transport**

Romel Somwar, Wenyan Niu, David Y. Kim, Gary Sweeney, Varinder K. Randhawa, Carol Huang, Toolsie Ramlal and Amira Klip

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