RESEARCH COMMUNICATION

Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin

James F. CLARKE,* Paul W. YOUNG,† Kazuyoshi YONEZAWA,‡ Masato KASUGA‡ and Geoffrey D. HOLMAN*§

*Department of Biochemistry, University of Bath, Bath BA2 7AY, U.K., †SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom, Surrey KT18 5XQ, U.K., and ‡Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-Cho, Chuo-Ku, Kobe 650, Japan

Wortmannin is a potent and reversible inhibitor of insulin-stimulated PtdIns 3-kinase activity in 3T3-L1 cells (IC_{50} = 2.6±0.8 nM). Wortmannin inhibits the PtdIns 3-kinase activity which is precipitated with antibodies against insulin receptor substrate 1 and against the α-p85 subunit of PtdIns 3-kinase. These observations suggest that wortmannin inhibits at the p110 catalytic subunit of PtdIns 3-kinase. Insulin stimulation of glucose transport in permeabilized 3T3-L1 cells is also inhibited by wortmannin (IC_{50} = 6.4±1.4 nM). Wortmannin did not inhibit basal glucose transport activity. The close similarity of the IC_{50} values for wortmannin inhibition of insulin-stimulated PtdIns 3-kinase and glucose transport activities suggests that the PtdIns 3-kinase is a key intermediate in insulin signalling of glucose-transport stimulation. The wortmannin inhibitory effect on transport is associated with a reduction in the cell-surface, but not the total cellular, levels of both GLUT1 and GLUT4 glucose transporter isoforms that are accessible to the cell-impermeant photolabel, ATB-BMPA. These photolabelling results suggest that the glucose transporter translocation process is dependent upon PtdIns 3-kinase activity. The stimulatory effect of guanosine 5′-[γ-thio]triphosphate (GTPγS) on glucose transport activity in permeabilized cells is only partially blocked by concentrations of wortmannin that completely inhibit the stimulatory effect of insulin. The residual stimulatory effect of GTPγS that occurs in the presence of wortmannin suggests that at least part of the GTPγS effect is mediated at a signalling site that is downstream of the site at which wortmannin inhibits the insulin stimulation of PtdIns 3-kinase and glucose transport activities.

INTRODUCTION

The stimulation by insulin of glucose transport activity in target tissues is mainly due to the translocation of the GLUT4 isoform from an intracellular vesicle pool to the plasma membrane [1,2]. The GLUT4 translocation process has been demonstrated in both adipose [3] and muscle [4] target tissues but also in the insulin-responsive cell line, 3T3-L1 [5]. The stimulation of GLUT4 translocation has been shown to be due to increased exocytosis of GLUT4 vesicles to the cell surface of both rat adipose cells [6] and 3T3-L1 cells [7], but the mechanism of signalling to the vesicle exocytosis process has not been studied in detail. A plausible candidate as a signalling intermediate between the insulin receptor and the GLUT4-vesicle translocation process is the enzyme PtdIns 3-kinase. The yeast homologue (VPS34) is known to be involved in vesicle budding and sorting processes [8]. Furthermore, the p110 catalytic subunit of PtdIns 3-kinase is now known to associate with insulin receptor substrate 1 (IRS1) through its associated α-p85 subunit. Two SH2 domains in α-p85 associate with a repeat YMXM motif in IRS1 following tyrosine phosphorylation by the insulin-receptor tyrosine kinase activity [9–13].

Evidence that suggests that IRS1-coupled PtdIns 3-kinase activity may be important in stimulation of glucose transport has been obtained in Chinese hamster ovary (CHO) cells transfected with the insulin receptor plus an α-p85 construct in which the p110-binding domain is deleted [13]. The presence of this construct prevents normal activation of IRS1-precipitatable PtdIns 3-kinase activity and also prevents insulin stimulation of glucose transport. This effect on transport has been attributed to a decreased GLUT1 translocation to the cell surface of the CHO cells, as detected using the bis-mannose photolabel, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA).

An important tool for investigation of the possible involvement of PtdIns 3-kinase in insulin stimulatory processes would be a potent and specific inhibitor of this enzyme. Wortmannin appears to be such a reagent, inhibiting PtdIns 3-kinase in the nanomolar concentration range but activities including phospholipase D, protein kinase C, cyclic GMP-dependent-, cyclic AMP-dependent- and calmodulin-dependent protein kinases, platelet-derived growth factor-receptor tyrosine kinase and myosin light-chain kinase (MLCK) in the micromolar concentration range [14]. Recently, Kanai et al. [15] have shown that wortmannin inhibits both PtdIns 3-kinase activity and GLUT4 translocation in CHO cells expressing the insulin receptor and a GLUT4 construct tagged by the *myc* epitope.

3T3-L1 cells are much more acutely sensitive to insulin than CHO cells so, in order to further demonstrate that the wortmannin inhibitory effect on glucose transport is associated with impairment of the glucose-transporter translocation process, we have studied GLUT1 and GLUT4 translocation in 3T3-L1 cells using the ATB-BMPA photolabelling technique [5,16]. The demonstration that, in a highly insulin-responsive cell line, GLUT1 and GLUT4 translocation is sensitive to wortmannin in a manner that correlates with inhibition of PtdIns 3-kinase activity adds to the evidence for PtdIns 3-kinase involvement in insulin signalling to the glucose transporter vesicle translocation

Abbreviations used: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GLUT, glucose transporter isoform; GTPγS, guanosine 5′-[γ-thio]triphosphate; ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine; IRS1, insulin receptor substrate 1; KRH, Krebs–Ringer–Hepes; MAP kinase, mitogen-activated protein kinase; MLCK, myosin light-chain kinase.

§ To whom correspondence should be addressed.

process. Very recently, Okada et al. have shown that wortmannin inhibits insulin stimulation of glucose transport activity and antilipolytic activity in rat adipocytes [17].

EXPERIMENTAL

Materials

ATB-[2-^{3}H]BMPA (sp. radioactivity approx. 10 Ci/mmol) was prepared as described [16], 2-deoxy-D-[2,6-^{3}H]glucose and [U-^{14}C]sucrose were from Amersham International, and [γ-^{32}P]ATP was from New England Nuclear. PtdIns was from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was from Flow Laboratories and fetal bovine serum from Gibco. Monocomponent insulin was a gift from Dr. Ronald Chance (Eli Lilly Corp., Indianapolis, IL, U.S.A.). Dexamethasone, isobutylmethylxanthine, Protein G–Sepharose, digitonin and GTPγS were from Sigma. Streptolysin O was from Murex Diagnostics. Thesit was from Boehringer.

Cell culture

3T3-L1 fibroblasts were obtained from the American Type Culture Collection, and were cultured in DMEM and differentiated to adipocytes by treatment with insulin, dexamethasone and isobutylmethylxanthine as described previously [5,18]. Fully differentiated cells were washed with PBS (154 mM NaCl, 12.5 mM sodium phosphate, pH 7.4) and were then incubated for 2 h in serum-free medium containing 25 mM D-glucose. This was followed by three washes in Krebs–Ringer–Hepes (KRH) buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM Hepes, pH 7.4) before use in experiments to determine 2-deoxy-D-glucose transport, cell-surface and total cell transporter activity and PtdIns 3-kinase activity.

Immunoprecipitation and assay of PtdIns 3-kinase

3T3-L1 cells in 35-mm-diam. dishes, after incubation either in the absence or presence of 100 nM insulin, were solubilized in 5 mM Na_2HPO_4, pH 7.2, containing 0.4 mM sodium orthovanadate, 1% $C_{12}E_9$ (Thesit), 1 μg/ml protease inhibitors and 1 mM dithiothreitol for 20 min. After centrifugation for 20 min at 20000 *g*, the supernatant was subjected to immunoprecipitation. Anti-IRS1 and anti-(α-p85) monoclonal antibodies (200 μl) were preabsorbed on to Protein G–Sepharose (30 μl) for 3 h at 4 °C. The Protein G–Sepharose conjugate was washed three times with 5 mM Na_2HPO_4, pH 7.2, and the solubilized cell supernatant added and incubated for 16 h at 4 °C with gentle rotation. The immune pellet was washed twice with 12.5 mM Na_2HPO_4, pH 7.2, 154 mM NaCl, 1% (w/v) Thesit, 1 mM dithiothreitol, twice with 0.1 mM Tris, pH 7.4, 0.5 M LiCl, 1 mM dithiothreitol and twice with 10 mM Tris, pH 7.4, 0.1 mM NaCl, 1 mM dithiothreitol.

The PtdIns 3-kinase activity was measured directly in immunoprecipitates in 50 μl samples containing 20 mM Hepes, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$ and 10 μg of PtdIns. The PtdIns was incubated with the immunoprecipitate for 5 min at room temperature and then 40 μM [γ-^{32}P]ATP (10 μCi) was added. The assay was stopped after 20 min by the addition of 30 μl of 4 M HCl and 130 μl of chloroform/methanol (1:1, v/v). The tubes were vortexed for 1 min, spun in a microfuge to separate the phases, and 20 μl of the lower phase was spotted on to a Silica Gel 60 plate that had been pretreated with 1% (w/v) potassium oxalate and activated at 100 °C for 1 h [9,11]. The t.l.c. resolving mixture was chloroform/methanol/water/ammonia (60:47:11.3:3.2, by vol.). The t.l.c. plates were dried and visualized by autoradiography.

Glucose transport activity

Differentiated adipocytes in 35-mm-diam. dishes were maintained at 37 °C either in the presence or absence of 100 nM porcine monocomponent insulin for 30 min. The cells were then incubated with 50 μM 2-deoxy-D-[2,6-^{3}H]glucose in 1 ml of KRH buffer at 37 °C for 5 min. Cells were then rapidly washed three times in KRH buffer at 0–4 °C, and the radioactivity was extracted into 1 ml of 0.1 M NaOH. For experiments in which 3T3-L1 cells were permeabilized, a modification of the method of Robinson et al. [19] was used. 3T3-L1 cells were washed three times with IC buffer (5 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$,$6H_2O$, 20 mM Hepes, 140 mM potassium glutamate, pH 7.2) instead of KRH buffer and incubated for 5 min in 1 ml of IC buffer containing 0.8 i.u. of streptolysin O to permeabilize the plasma membranes. The cells were then washed a further three times with IC buffer and incubated in the presence or absence of insulin or GTPγS in IC buffer containing 3 mM sodium pyruvate and 10 mM ATP. The cells were then incubated for 5 min with 50 μM 2-deoxy-D-glucose (0.3 μCi of 2-deoxy-D-[2,6-^{3}H]glucose) and 0.06 μCi [U-^{14}C]sucrose, which was used to correct the tritium counts for non-specific diffusion of the label into the cells. Cells were then rapidly washed once in IC buffer at 0–4 °C, and the radioactivity extracted into 1 ml of 0.1 M NaOH.

ATB-BMPA labelling

Cells in 35-mm-diam. dishes were maintained at 37 °C in the absence or presence of 100 nM insulin for 30 min. The dishes were washed in KRH buffer and were irradiated for 1 min in a Rayonet photochemical reactor in the presence of 100 μCi of ATB-[2-^{3}H]BMPA in 250 μl of KRH buffer at 18 °C. To measure labelling of the total cellular transporter pool, cells were permeabilized by treatment with 0.025% (w/v) digitonin for 8 min at 18 °C in the presence of 100 μCi ATB-[2-^{3}H]BMPA [20]. The irradiated cells were washed four times in KRH buffer and solubilized in 1.5 ml of detergent buffer containing 2% Thesit, 5 mM sodium phosphate and 5 mM EDTA, pH 7.2, and the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin each at 1 μg/ml. After centrifugation at 20000 *g* for 20 min, the detergent-solubilized samples were subjected to sequential immunoprecipitation with 30 μl of Protein A–Sepharose coupled with 100 μl of anti-GLUT1 or 50 μl of anti-GLUT4 serum. The antisera were raised against peptides corresponding in sequence to the GLUT1 and GLUT4 C-terminal segments [20]. After incubation for 2 h at 0–4 °C, the immunoprecipitates were washed three times with 1.0%, and then once in 0.1%, Thesit detergent buffer. Labelled glucose transporters were then released from the antibody complexes with 10% (w/v) SDS/6 M urea/10% (v/v) mercaptoethanol and subjected to electrophoresis on 10% (w/v) acrylamide gels. The radioactivity on the gel was extracted from the gel slices and estimated by liquid-scintillation counting [20].

RESULTS

Effects of wortmannin on PtdIns 3-kinase activity

We have examined the effects of wortmannin on PtdIns 3-kinase activity in both α-p85 and IRS1 immunoprecipitates (Table 1) to determine whether wortmannin alters the coupling of PtdIns 3-kinase to IRS1. Wortmannin inhibits α-p85- and IRS1-precipitated PtdIns 3-kinase activity to an equal extent, supporting the suggestion [14] that wortmannin interacts with the 110 kDa

Table 1 Effect of wortmannin on IRS1- and α-p85-precipitated PtdIns 3-kinase activity

3T3-L1 cells were incubated in the absence or presence of 100 nM insulin, solubilized in $C_{12}E_9$ detergent buffer and then the PtdIns 3-kinase activity was precipitated using either an α-p85 or an IRS1 monoclonal antibody preabsorbed with Protein G–Sepharose. The PtdIns 3-kinase activity in the immunoprecipitates was determined either in the absence or presence of 1 μM wortmannin. Results are expressed as a percentage of the activity (mean ± S.E.M., $n = 3$) obtained from the insulin-treated cells.

Immunoprecipitation	3T3-L1 cell treatment	PtdIns 3-kinase activity (%)
α-p85	Insulin	100
	Basal	47.6 ± 7.8
	Insulin + 1 μM wortmannin in PtdIns 3-kinase assay	2.7 ± 0.7
	Insulin + 1 μM wortmannin in washed cells	65.7 ± 25.5
IRS1	Insulin	100
	Basal	12.6 ± 4.1
	Insulin + 1 μM wortmannin in PtdIns 3-kinase assay	1.7 ± 0.5
	Insulin + 1 μM wortmannin in washed cells	53.9 ± 24.2

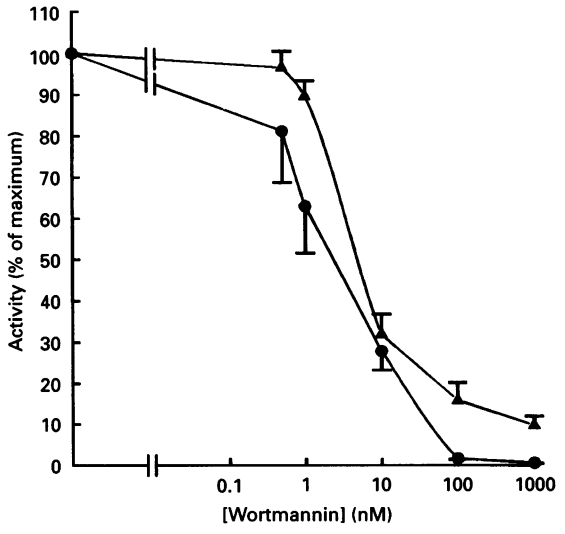

Figure 1 Inhibition of PtdIns 3-kinase and glucose transport activity by wortmannin

3T3-L1 cells in 35-mm-diam. dishes were treated with the indicated concentrations of wortmannin and 100 nM insulin and then either glucose transport activity was determined using 2-deoxy-D-glucose as substrate (▲) or the cells were dissolved in $C_{12}E_9$ detergent buffer for precipitation with IRS1 antibody. The indicated concentrations of wortmannin were then added to the assay for PtdIns 3-kinase activity (●). Results are means ± S.E.M. of three experiments.

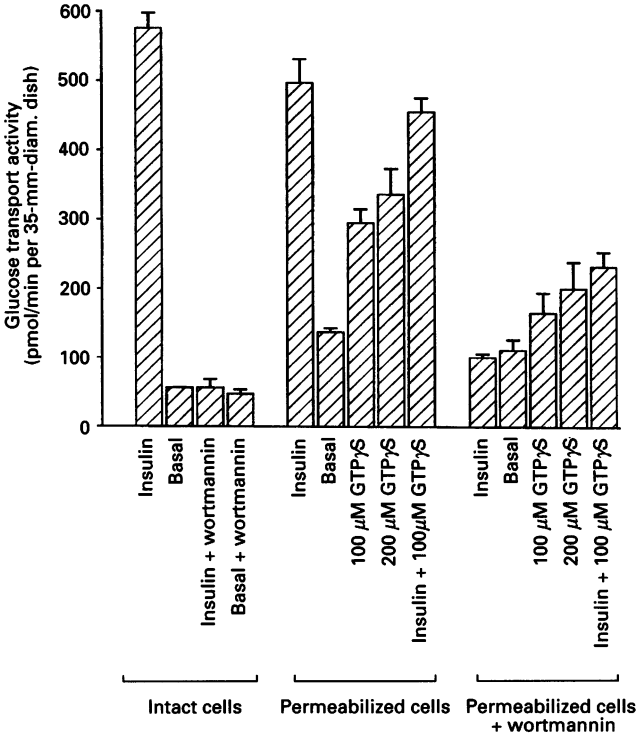

Figure 2 Comparison of the inhibitory effects of wortmannin on insulin- and GTPγS-stimulated glucose transport activity

3T3-L1 cells were either maintained in the absence or presence of 100 nM insulin and, where indicated, 1 μM wortmannin. The effect of GTPγS was determined in streptolysin O-permeabilized cells. Glucose transport activity was determined using 2-deoxy-D-glucose as the substrate. Results are the means ± S.E.M. of three experiments.

subunit of PtdIns 3-kinase. When intact cells are treated with wortmannin, and then PtdIns 3-kinase is subsequently measured in detergent extracts of washed cells, the activity is higher than that observed when wortmannin is added directly to the PtdIns 3-kinase assay mixture (Table 1). This is probably a dilution or inactivation effect, but suggests that the inhibitory effect of wortmannin is partly reversible and is lost following extensive washing of the 3T3-L1 cells and immunoprecipitates, which removes or inactivates the inhibitor.

Inhibition of glucose transport activity

The concentrations of wortmannin that are required to inhibit insulin-stimulated PtdIns 3-kinase activity and glucose transport activity are compared in Figure 1. The IC_{50} values for inhibition of PtdIns 3-kinase and glucose transport activity are very similar and are found to be 2.6 ± 0.8 nM (from three experiments) and 6.4 ± 1.4 nM (from three experiments) respectively. Wortmannin did not inhibit basal glucose transport activity, either in intact cells or permeabilized cells (where the basal glucose transport activity is elevated as a consequence of the manipulations involved in the permeabilization procedure) (Figure 2).

In order to resolve further the site of action of wortmannin in the insulin stimulation cascade, we examined the effect of wortmannin on GTPγS-stimulated glucose transport activity. In permeabilized cells, 100–200 μM GTPγS stimulates glucose trans-

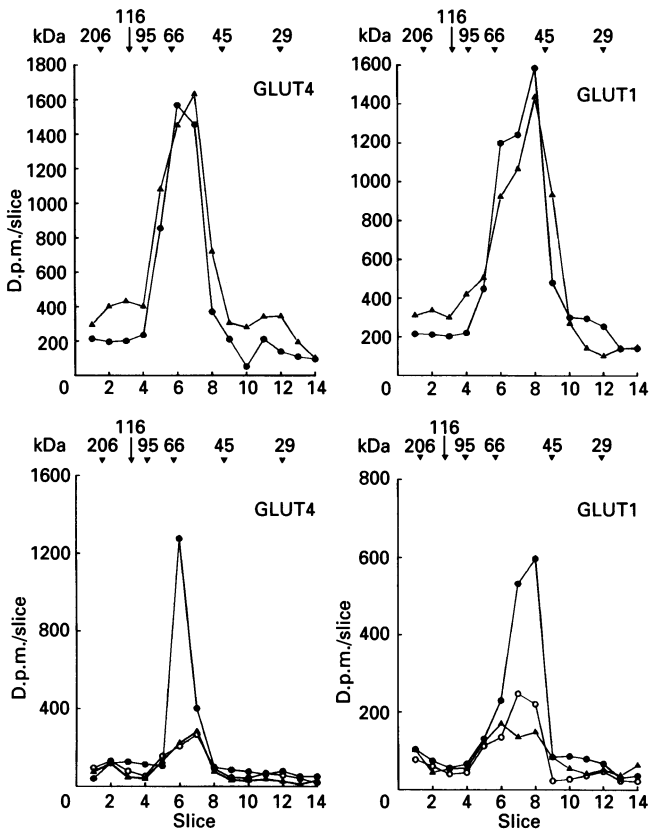

Figure 3 Wortmannin inhibition of glucose transporter translocation as detected using the ATB-BMPA photolabel

3T3-L1 cells were maintained in the absence (○) or presence (●, ▲) of 100 nM insulin. The total cellular and cell-surface levels of GLUT4 and GLUT1 were determined by photolabelling with ATB-BMPA in digitonin-permeabilized cells (**a**) or in intact cells (**b**) respectively, either in the absence or presence (▲) of 1 μM wortmannin. The labelled transporters were separated by immunoprecipitation with anti-(C-terminal peptide) antibodies and resolved by gel electrophoresis. The results are from a single experiment representative of three similar experiments.

port activity by approx. 2-fold (Figure 2). This is consistent with results obtained by Robinson et al. [19], who suggested that the GTP effect is mediated at a late stage in the process of stimulation of glucose transporter translocation. If the site of wortmannin action is upstream of this late site of action of GTP in the stimulatory process, then wortmannin should be less effective as an inhibitor of the stimulation by GTP than that by insulin. We have found that in the presence of 1 μM wortmannin, GTPγS is still able to partially stimulate glucose transport activity, whereas this concentration of wortmannin completely inhibits the insulin stimulation of transport activity (Figure 2). The level of transport stimulation by GTPγS is less in the presence than in the absence of wortmannin, suggesting that not all of the stimulatory effect of GTPγS is downstream of the site of wortmannin action. In addition, when insulin and GTPγS are added together with wortmannin a glucose transport stimulation occurs, producing a level of transport activity that is similar to that observed with GTPγS alone.

Inhibition of glucose transporter translocation

In 3T3-L1 cells both the GLUT1 and GLUT4 isoforms are present in roughly equal amounts [20]. Insulin can induce a 3–5-fold stimulation of translocation of GLUT1 but a much greater, 10–15-fold, stimulation of translocation of the GLUT4 isoform. The impermeant photolabel, ATB-BMPA, has therefore been used to assess whether the observed inhibition of glucose transport activity by wortmannin is associated with reduced translocation of either, or both, of these isoforms. In order to show that wortmannin does not interfere with the glucose transporters directly, we measured the binding of ATB-BMPA in digitonin-permeabilized cells. Figure 3(a) shows that in digitonin-permeabilized cells, the labelling of neither GLUT1 nor GLUT4 is altered by wortmannin. Instead, the wortmannin clearly reduces the amounts of these isoforms at the cell surface. Figure 3(b) shows the labelling of these isoforms in intact cells that are fully stimulated by treatment with 100 nM insulin. The levels of labelling observed in the wortmannin-treated samples are reduced to basal levels.

DISCUSSION

Recently, the complex process of dissecting out signalling intermediates between the insulin receptor and the glucose transporter translocation machinery has made some important advances. Several potential sources of signal emanating from the tyrosine phosphorylation of IRS1 have been suggested by Myers and White [10]. The common feature of the signalling systems that diverge at the level of IRS1 is the involvement of docking proteins containing SH2 domains. Downstream insulin signalling through Ras occurs through interaction of the GTP-exchange protein GRB-2 with IRS1 [21]. Ras is thought to initiate activity in a sequential cascade of serine kinases initially involving Raf, mitogen-activated protein (MAP) kinase kinase, MAP kinase and then MLCK and 90K S6 kinase among others [10,21]. The p110 catalytic subunit of PtdIns 3-kinase is coupled to IRS1 via α-p85 [9,10]. The homologous β-p85 may be coupled to a downstream enzyme in a manner analogous to the coupling of α-p85 to p110 [10]. In addition, signalling from IRS1 may involve the tyrosine phosphatase Syp [22], or perhaps some as yet unidentified docking protein containing SH2 domains.

The two potential signalling routes that have been most studied in relation to glucose transport activation are the Ras-activated MAP kinase system and the PtdIns 3-kinase system. Although changes in glucose transport occur in response to elevation of Ras [23,24] and MAP kinase [25], these effects may be due to increased transcription [23,24] and translocation [25] of GLUT1. Evidence against the involvement of MAP kinase activation in the acute insulin stimulation of glucose transport activity has been obtained by comparing the effects of insulin and epidermal growth factor (EGF) on MAP kinase with the corresponding effects on glucose transport stimulation. While EGF and insulin both stimulate MAP kinase phosphorylation [26,27], EGF has only a very small effect on glucose transport and GLUT4 translocation in rat adipocytes [28] and on glucose transport in 3T3-L1 cells [26], attributed to GLUT1 translocation [27]. It has been suggested that the reagent ML9, through inhibition of MAP kinase phosphorylation, inhibits glucose transport activation by insulin in 3T3-L1 cells [29]. However, ML9 is not specific for MAP kinase kinase and may also inhibit PtdIns 3-kinase [15]. The insulin-dependent activation of 70K S6 kinase has been shown not to be necessary for glucose transport stimulation [30].

The possibility that glucose transport activation occurs through coupling of PtdIns 3-kinase activity to IRS1 has recently been explored [13,15,17]. Evidence presented here supports this possibility as we have shown a clear correspondence between the ability of wortmannin to inhibit PtdIns 3-kinase and glucose

transporter translocation. If the basis of the wortmannin inhibition of glucose transport translocation is at the level of PtdIns 3-kinase then our results suggest that at least part of the GTPγS stimulatory effect is downstream of this site. GTPγS may stimulate, even in the presence of wortmannin, by interaction with a small G-protein involved in vesicle trafficking.

The availability of a reagent that potently (at nanomolar concentrations) inhibits the signalling to glucose transport translocation is unprecedented. Since wortmannin (at nanomolar concentrations) probably interacts specifically at PtdIns 3-kinase it may continue to be a useful tool in the dissection of signalling steps in glucose transport activation. The use of this reagent has added support to the hypothesis that PtdIns 3-kinase is a key intermediate in the signalling cascade leading to glucose transport stimulation [13,15,17]. The attraction of this hypothesis is that PtdIns 3-kinase is known to be involved in other membrane trafficking events, although it is evident that, at least in the analogous yeast VPS34 system, the PtdIns 3-kinase operates in conjunction with a protein kinase in controlling membrane trafficking events [8]. It has recently been shown [31] that wortmannin is a potent inhibitor of histamine release from a basophil cell line, and it seems likely that this effect is also mediated by inhibition of PtdIns 3-kinase activity. Such findings add growing support for the hypothesis that this enzyme will be found to be a key intermediate in many regulated vesicle-trafficking processes.

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