Disruption of GLUT1 glucose carrier trafficking in L6E9 and Sol8 myoblasts by the phosphatidylinositol 3-kinase inhibitor wortmannin

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In this study we have used wortmannin, a highly specific inhibitor of phosphatidylinositol (PI) 3-kinase, to assess the role of this enzyme on GLUT1 glucose carrier distribution and glucose transport activity in myoblasts from two skeletal-muscle cell lines, L6E9 and Sol8. As detected in L6E9 cells, myoblasts exhibited basal and insulin-stimulated PI 3-kinase activities. Incubation of intact myoblasts with wortmannin resulted in a marked inhibition of both basal and insulin-stimulated PI 3-kinase activities. L6E9 and Sol8 myoblasts showed basal and insulin-stimulated glucose transport activities, both of them inhibited by wortmannin in a dose-dependent manner ($IC_{50} \approx 10\text{--}20\text{ nM}$). Concomitantly, immunofluorescence analysis revealed that 1 h treatment with wortmannin led to a dramatic intracellular accumulation of GLUT1 carriers (the main glucose

transporter expressed in L6E9 and Sol8 myoblasts) in both cell systems. The effect of wortmannin on GLUT1 cellular redistribution was independent of the presence of insulin. The cellular distribution of two structural plasma-membrane components such as β_1 -integrin or the α_1 subunit of the Na^+K^+ -ATPase were unaffected by wortmannin in both the absence and the presence of insulin. As a whole, our results indicate that PI 3-kinase is necessary to basal and insulin-stimulated glucose transport in L6E9 and Sol8 myoblasts. Moreover, immunofluorescence assays suggest that in both cellular models there is a constitutive GLUT1 trafficking pathway (independent of insulin) that involves PI 3-kinase and which, when blocked, locks GLUT1 in a perinuclear compartment.

INTRODUCTION

Recent biochemical data have improved our understanding of the signal-transduction pathways leading to insulin-stimulated glucose transport and glucose transporter translocation. During the last 2 years, phosphatidylinositol (PI) 3-kinase has emerged as a critical step for such events in CHO cells, isolated brown and white adipocytes and 3T3 L1 adipose cells [1–6].

Important tools for most of these studies have been the recently described highly specific inhibitors of PI 3-kinase, wortmannin and LY294002 [7,8]. Wortmannin has been shown to inhibit the lipid and serine kinase activity of PI 3-kinase in the nanomolar concentration range [9,10]. However, some recent reports have described different isoforms of PI 3-kinase which are less sensitive to wortmannin. The currently characterized mammalian PI 3-kinase is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit [11,12]. There are several regulatory ways proposed for PI 3-kinase activity (reviewed in [13]), one of them being the binding of p85-SH2 domains to tyrosine-phosphorylated proteins such as the insulin receptor substrate-I [14–20]. Partially purified PI 3-kinase is inhibited by wortmannin with an IC_{50} of about 4 nM [21]. In contrast, the yeast Vps34 gene product PI 3-kinase activity and the PI-specific PI 3-kinase from bovine cells are inhibited at concentrations above 1 and 0.1 μM wortmannin, respectively [21,22]. Curiously enough, this latter PI 3-kinase is not activated by phosphotyrosine-containing peptides from insulin receptor substrate-I or platelet-derived growth factor receptor. Recently, a novel mammalian PI 3-kinase has been described which is activated by G-protein $\beta\gamma$ subunits and inhibited by wortmannin with an IC_{50} of nearly 200 nM [23].

In CHO cells, smooth-muscle cells, platelets, neutrophils, T-cells and rat adipocytes, PI 3-kinase activation has been shown to increase PI 3,4-bisphosphate and 3,4,5-trisphosphate [$PI(3,4)P_2$ and $PI(3,4,5)P_3$], with little change in PI 3-monophosphate [$PI(3)P$] [20,24–27]. For this reason, it has been suggested that $PI(3,4)P_2$ and $PI(3,4,5)P_3$, but not $PI(3)P$, may mediate the signalling induced by PI 3-kinase activation. Indeed, very recently, Toker et al. [28] have reported that $PI(3,4)P_2$ and $PI(3,4,5)P_3$ could act as second messengers to activate PKC δ , ϵ or η *in vivo*. However, in intact soleus muscle incubated with [^{32}P]P, insulin increased the labelling of $PI(3)P$ without any detectable production of $PI(3,4)P_2$ or $PI(3,4,5)P_3$ [29]. These observations raise important questions regarding the role of each lipid product of PI 3-kinase in the regulation of distinct cellular functions. In this context, data accumulated lead to the notion that $PI(3,4)P_2$ and $PI(3,4,5)P_3$ may act as second messengers to signal cell proliferation in response to growth factor stimulation [30], whereas $PI(3)P$ would be specifically involved in regulating intracellular protein-sorting processes [22].

In this paper, we have used wortmannin to assess the role of PI 3-kinase in GLUT1 glucose carrier distribution and glucose transport activity in myoblasts from two skeletal-muscle cell lines, L6E9 and Sol8. In contrast with what is reported for adipose tissue, 3T3 L1 cells, CHO cells and L6 myotubes, where wortmannin affected only the insulin-dependent responses [2,4,6,31], our results indicate that, in L6E9 and Sol8 myoblasts, PI 3-kinase may be essential for GLUT1-dependent basal as well as insulin-stimulated glucose transport. Immunofluorescence assays showed that, in both cell models, there is a constitutive GLUT1 trafficking pathway, not dependent on insulin, which is disrupted by wortmannin.

Abbreviations used: PI, phosphatidylinositol; $PI(3)P$, PI 3-monophosphate; $PI(4)P$, PI 4-monophosphate; $PI(3,4)P_2$, PI 3,4-bisphosphate; $PI(3,4,5)P_3$, PI 3,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate.

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MATERIALS AND METHODS

Materials

PI, PI 4-monophosphate [PI(4)P] and Hoechst 33342 were from Sigma (St. Louis, MO, U.S.A.). [γ - 32 P]ATP was from ICN (Costa Mesa, CA, U.S.A.). 2-Deoxy[3 H]glucose was obtained from New England Nuclear. Wortmannin was kindly given by Dr. Trevor Payne (Sandoz, Basel, Switzerland). The polyclonal antibodies against rat p85 PI 3-kinase and against the α_1 subunit of the Na $^+$ -K $^+$ -ATPase were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). A polyclonal antibody generated against the C-terminus of GLUT1 was obtained from Biogenesis Inc. (Sandown, NH, U.S.A.). A rabbit polyclonal antibody against β_1 -integrin was kindly given by Dr. Carles Enrich (University of Barcelona) [32]. Sol8 and L6E9 rat skeletal-muscle cell lines were kindly provided by Dr. C. Pinset (Institut Pasteur, Paris, France) and Dr. B. Nadal-Ginard (Harvard University, U.S.A.) respectively. Dulbecco's modified Eagle's medium (DMEM), fetal-bovine serum and pancreatin were obtained from Whittaker (Walkersville, MD, U.S.A.) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig was from Dakopatts (Glostrup, Denmark). TLC silica gel 60A was obtained from Whatman.

Cell culture

Skeletal-muscle cell lines Sol8 and L6E9, from neonatal mouse and rat respectively, were grown in monolayer culture in DMEM containing 10% (v/v) fetal-bovine serum and 1% (v/v) antibiotics (10000 units/ml penicillin G and 10 mg/ml streptomycin). Myoblasts incubated for short times with wortmannin and/or insulin were first depleted of serum by prior incubation for 2 h in DMEM containing 0.2% BSA. For immunofluorescence studies, cells were grown under the same conditions on glass coverslips.

PI 3-kinase activity

Before PI 3-kinase assay, cells were incubated in DMEM containing 0.2% BSA for 2 h. Cells were then treated (or not) with 1 μ M wortmannin for 30 min at 37 °C before the addition (or not) of insulin to a final concentration of 1 μ M, followed by a further incubation for 30 min at 37 °C. After washing twice in PBS, cells were scraped and solubilized for 30 min at 4 °C in a buffer containing 50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na $_4$ P $_2$ O $_7$, 100 mM NaF, 2 mM vanadate, 0.5 mM PMSF, 2 mM leupeptin and 2 mM pepstatin, supplemented with 1% Nonidet P40. The solubilized preparations were centrifuged at 10000 *g* for 20 min at 4 °C, and 2.5 mg of each supernatant was immunoprecipitated with 5 μ l of anti-p85 antiserum or non-immune controls (results not shown). Antibodies were pre-adsorbed on Protein G-Sepharose at 4 °C for 1 h and washed twice in 30 mM Hepes/30 mM NaCl/0.1% Triton X-100, pH 7.4, before being incubated with the solubilized proteins for 90 min at 4 °C. As described previously [17], the immunoprecipitates were washed twice in each of the following buffers: (a) PBS, pH 7.4, containing 1% Nonidet P40; (b) 100 mM Tris/0.5 M LiCl, pH 7.4; and (c) 10 mM Tris/100 mM NaCl/1 mM EDTA, pH 7.4. The pellets were resuspended in 30 μ l of 20 mM Hepes/0.4 mM EDTA/0.4 mM Na $_2$ HPO $_4$. The substrate (PI) was dried for 10 min, resuspended in 5 mM Hepes at 1 mg/ml and sonicated for 15 min. The kinase reaction was started by the addition of PI at a final concentration of 0.2 mg/ml, 10 mM MgCl $_2$ and 50 μ M [γ - 32 P]ATP (10 Ci/mmol). After 15 min the reaction was stopped by addition of 15 μ l of 4 M HCl, and the phosphoinositol lipids were extracted with 130 μ l of chloroform/methanol (1:1, v/v). The tubes were mixed by

vortexing for 30 s and centrifuged for 1 min. The organic phase was recovered and dried, and the phospholipids were resuspended in 5 μ l of chloroform and separated by TLC on silica gel 60A in chloroform/methanol/4.3 M ammonia (9:7:2, by vol.). The plate was analysed by autoradiography.

Glucose uptake

Before transport experiments, cells were incubated for 2 h in DMEM containing 0.2% BSA. Cells were then treated (or not) with wortmannin at the concentrations indicated in each case for 30 min at 37 °C before adding (or not) 1 μ M insulin for a further 30 min. Cells were then washed, and transport solution (20 mM Hepes, 150 mM NaCl, 5 mM KCl, 5 mM MgSO $_4$, 1.2 mM KH $_2$ PO $_4$, 2.5 mM CaCl $_2$, 2 mM pyruvate, pH 7.4) was added, together with 100 μ M 2-deoxy-D-[3 H]glucose (96 mCi/mmol). After 20 min, transport was stopped by addition of 2 vol. of ice-cold 50 mM glucose in PBS. Cells were washed three times in the same solution and disrupted with 0.1 M NaOH/0.1% SDS. Radioactivity was determined by scintillation counting. Protein was determined by the Bradford method [33]. Each condition was run in triplicate, and the non-specific uptake ($t = 0$) was determined by incubation of the 2-deoxy-D-[3 H]glucose in stop solution (50 mM glucose in PBS) instead of transport solution. In all cases, the value at $t = 0$ represented 4% of the basal transport activity at $t = 20$ min. Glucose transport under basal and stimulated conditions was linear over the time period assayed (results not shown).

Immunofluorescence analysis

For immunofluorescence labelling [34], cells were grown on glass coverslips. Cells were starved for 2 h in DMEM containing 0.2% BSA and then treated (or not) with 0.1 or 1 μ M wortmannin for 1 h. When indicated, 1 μ M insulin was added during the last 30 min of incubation. Coverslips were then rinsed in PBS, fixed with methanol (–20 °C) for 2 min, washed twice with PBS and processed. Cells fixed on coverslips were incubated with 30 μ l of primary antibody [1/100 dilution of anti- β_1 -integrin, 1/50 dilution of anti-(α_1 subunit of the Na $^+$ -K $^+$ -ATPase), 1/50 dilution of anti-GLUT1 in PBS containing 0.5% BSA] for 45 min at 37 °C. Coverslips were washed three times in PBS, the last one for 15 min, before incubation with the secondary antibody (1/50 dilution of FITC-conjugated goat anti-rabbit immunoglobulins in PBS containing 0.5% BSA) for 30 min at 37 °C. Coverslips were then washed three times in PBS, the last one for 15 min and in the presence of nuclear stain Hoechst 33342. Finally, coverslips were mounted with immunofluorescence medium and viewed with an $\times 40$ objective using a Reichert Jung Polyvar II microscope equipped with epifluorescence illumination. Photographic exposures were made with Kodak Tmax 400 film.

RESULTS

Wortmannin inhibits basal and insulin-stimulated PI 3-kinase activity in intact myoblasts

Myoblasts of the L6E9 rat skeletal-muscle cell line were incubated with wortmannin and/or insulin as indicated. PI 3-kinase was immunoprecipitated with an antiserum to the p85 subunit from solubilized cells and kinase activity was assayed with PI as a substrate. Myoblasts exhibited basal PI 3-kinase activity which was stimulated 2.1 ± 0.6 ($n = 3$)-fold in insulin-treated cells (Figure 1). When intact L6E9 myoblasts were incubated with wortmannin, a marked decrease in PI 3-kinase was detected, both in the absence and in the presence of insulin (Figure 1). As seen in Figure 1 for the insulin-treated myoblasts, the effect of

Figure 1 Wortmannin inhibits both basal and insulin-stimulated PI 3-kinase activity in L6E9 muscle cells

L6E9 myoblasts were serum starved and then treated (or not) with wortmannin for 1 h at the indicated concentrations. During the last 30 min of incubation, cells were stimulated with 1 μ M insulin (+) or left untreated (—). Cells were solubilized, and proteins (2.5 mg) were incubated with 5 μ l of an antiserum recognizing the p85 subunit of PI 3-kinase (Upstate Biotechnology Inc.), and Protein G-Sepharose. Immune complexes were analysed for PI 3-kinase activity as described in the Materials and methods section, using PI and [γ - 32 P]ATP as substrates. A representative autoradiograph of the TLC is shown.

wortmannin was dose-dependent, with an almost total suppression of PI 3-kinase activity at a concentration of 100 nM. The product of the PI kinase reaction catalysed by the anti-p85 immunoprecipitates was PI(3)P only; no PI(4)P was generated during the incubation, indicating that the immunoprecipitates were not contaminated with PI 4-kinase (results not shown). Thus, myoblasts exhibited basal and insulin-stimulated PI 3-kinase activity, both of which were inhibited by wortmannin.

Wortmannin inhibits basal and insulin-stimulated 2-deoxyglucose uptake in muscle cells

We studied the effect of wortmannin on 2-deoxyglucose uptake under basal and insulin-stimulated conditions in L6E9 and Sol8 myoblasts. L6E9 and Sol8 myoblasts express GLUT1 as the main glucose carrier isoform, and myogenesis leads to the induction of GLUT4 and GLUT3 in L6E9, but only GLUT4 in Sol8 (F. Viñals, T. Santalucía and P. Kaliman, unpublished work). In the absence of wortmannin, L6E9 myoblasts responded to insulin by stimulating the rate of 2-deoxyglucose uptake by 63 % (Figure 2a). The presence of wortmannin caused a dose-dependent inhibition of basal and insulin-stimulated 2-deoxyglucose uptake in L6E9 myoblasts (Figure 2a). The concentration of wortmannin causing a 50 % inhibition of basal or insulin-stimulated glucose transport was approx. 10 nM and 20 nM for basal and insulin-stimulated glucose transport, respectively. These values are comparable with those previously found in other cell types, such as adipocytes or *Xenopus* oocytes [3,35].

The effect of wortmannin on basal and insulin-stimulated glucose transport was also evaluated in Sol8 myoblasts, a mouse skeletal-muscle cell line. In keeping with the observations in rat L6E9 cells, Sol8 myoblasts responded to insulin by stimulation of 2-deoxyglucose uptake, and wortmannin inhibited both basal and insulin-stimulated glucose transport in a dose-dependent manner (Figure 2b).

Wortmannin induces a major cellular redistribution of GLUT1 glucose carriers in myoblasts

On the basis of the inhibitory effect of wortmannin on glucose transport activity in muscle cells, we aimed to determine, in the

Figure 2 Inhibition by wortmannin of basal and insulin-stimulated 2-deoxyglucose uptake by L6E9 and Sol8 myoblasts

After serum starvation, L6E9 (a) and Sol8 (b) myoblasts were incubated for 1 h at various concentrations of wortmannin in the absence (black symbols or black bars) or in the presence of 1 μ M insulin (white symbols or white bars). After addition of 0.1 mM 2-deoxy[3 H]glucose, the cellular hexose uptake at $t = 20$ min was measured as described in the Materials and methods section. (a) and (b) show the means of three independent experiments where each point was done in triplicate.

next step, whether this effect of wortmannin was a consequence of the redistribution of glucose carriers. In order to answer this question, L6E9 and Sol8 myoblasts were incubated for 1 h in the absence or presence of wortmannin with the addition (or not) of insulin during the last 30 min of incubation. The distributions of β_1 -integrin and the α_1 subunit of the Na $^+$ -K $^+$ -ATPase (plasma-membrane markers) and GLUT1 were then determined by immunofluorescence analysis.

In L6E9 myoblasts, a substantial proportion of GLUT1 was distributed on the cell surface, on the basis of the similar immunofluorescence pattern found for β_1 -integrin, the α_1 subunit of the Na $^+$ -K $^+$ -ATPase and GLUT1 (Figures 3a, 3d and 4). Indeed, all three antibodies used clearly delineated the cell borders. Under our experimental conditions, we were not able to detect any effect of insulin compatible with a process of GLUT1 translocation to the cell surface. After exposure to wortmannin, most GLUT1 was found intracellularly, and a marked decrease in the immunofluorescence associated with the cell surface was detected both in the absence and in the presence of insulin (Figure 3: without insulin, panels a versus b and c; with insulin, panel d versus e). The effect of wortmannin on GLUT1 distribution was already maximal at concentrations of 0.1 μ M wortmannin both in the absence and in the presence of insulin (Figure 3b, and results not shown, respectively). In contrast with what was observed for GLUT1, no effect of 1 μ M wortmannin on the cellular distribution of the α_1 subunit of the Na $^+$ -K $^+$ -ATPase (Figure 4, a versus b) or β_1 -integrin (Figure 4, c versus

Figure 3 Wortmannin induces a cellular redistribution of GLUT1 in L6E9 myoblasts

Serum-starved L6E9 myoblasts were incubated in the absence or in the presence of wortmannin for 1 h. When indicated, 1 μ M insulin was added during the last 30 min of incubation. Cells were fixed with methanol and incubated with anti-GLUT1 antibody. Finally, cells were incubated with a FITC-conjugated secondary antibody, as described in the Materials and methods section. Scale bars represent 60 μ m (the scale is the same for all panels). Panels (a), (b) and (c): 0, 0.1 and 1 μ M wortmannin, respectively, in the absence of insulin. Panels (d) and (e): 0 and 1 μ M wortmannin, respectively, in the presence of insulin.

Figure 4 Wortmannin does not affect the cellular distribution of the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ or β_1 -integrin in L6E9 myoblasts

Serum-starved L6E9 myoblasts were incubated in the absence (panels a and c) or in the presence of 1 μ M wortmannin (panels b and d) for 1 h. Cells were fixed with methanol and incubated with antibody to the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (panels a and b) or with anti- β_1 -integrin antibody (panels c and d). Finally, cells were incubated with a FITC-conjugated secondary antibody, as described in the Materials and methods section. Scale bars represent 60 μ m (the scale is the same for all panels).

d) was detected. Figure 4 shows results obtained in the absence of insulin; identical results for α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ or β_1 -integrin were obtained in the presence of 1 μ M insulin (results not shown).

Immunofluorescence results in Sol8 myoblasts totally coincided with the results obtained in L6E9 myoblasts: (i) GLUT1 was detected on the cell surface, and no effect of insulin on GLUT1 distribution could be observed under our experimental conditions (Figures 5a and 5d); (ii) wortmannin caused a redistribution of GLUT1 from the cell surface to an intracellular perinuclear locus (Figure 5: without insulin, panels a versus b and c; with insulin, panel d versus e); (iii) the maximal effect of wortmannin was already detected at 0.1 μ M concentration (Figure 5b); (iv) no effect of 1 μ M wortmannin on the cellular distribution of the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Figure 6, a versus b) or β_1 -integrin (Figure 6, c versus d) was detected. Figure 6 shows results obtained in the presence of insulin; similar results (not shown) for these two plasma-membrane markers were obtained in the absence of insulin.

In summary, immunofluorescence data from L6E9 and Sol8 myoblasts suggest that PI 3-kinase inhibition by wortmannin affects a GLUT1 trafficking pathway that is already active in the absence of insulin. Furthermore, the alteration of the intracellular distribution of GLUT1 by wortmannin appears to be specific, since no change in the distribution of two cell-surface markers such as the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ and β_1 -integrin could be detected after wortmannin treatment.

DISCUSSION

In this paper we have analysed the effect of the highly specific inhibitor of PI 3-kinase, wortmannin, on glucose transport and GLUT1 glucose carrier trafficking in myoblasts from two skeletal-muscle cell lines, L6E9 and Sol8. L6E9 myoblasts exhibited basal and insulin-sensitive PI 3-kinase activities which were inhibited *in vivo* by wortmannin, as detected in solubilized cell preparations immunoprecipitated with anti-p85 antibodies, using PI as a substrate. In contrast with what is reported for other cell types, such as white and brown adipocytes [2,4] and

Figure 5 Wortmannin induces a cellular redistribution of GLUT1 in Sol8 myoblasts

Serum-starved Sol8 myoblasts were incubated in the absence or in the presence of wortmannin for 1 h. When indicated, 1 μ M insulin was added during the last 30 min of incubation. Cells were fixed with methanol and incubated with anti-GLUT1 antibody. Finally, cells were incubated with a FITC-conjugated secondary antibody, as described in the Materials and methods section. Scale bars represent 60 μ m (the scale is the same for all panels). Panels (a), (b) and (c): 0, 0.1 and 1 μ M wortmannin, respectively, in the absence of insulin. Panels (d) and (e): 0 and 1 μ M wortmannin, respectively, in the presence of insulin.

Figure 6 Wortmannin does not alter the cellular distribution of the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ or β_1 -integrin in Sol8 myoblasts

Serum-starved Sol8 myoblasts were incubated in the absence (panels a and c) or in the presence of 1 μ M wortmannin (panels b and d) for 1 h, and 1 μ M insulin was added during the last 30 min of incubation. Cells were fixed with methanol and incubated with antibody to the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (panels a and b) or with anti- β_1 -integrin antibody (panels c and d). Finally, cells were incubated with a FITC-conjugated secondary antibody, as described in the Materials and methods section. Scale bars represent 60 μ m (the scale is the same for all panels).

3T3-L1 cells [3], in which wortmannin altered only the insulin-stimulated component of glucose transport, myoblasts of both cell lines studied here (L6E9 and Sol8) responded to wortmannin

by decreasing both the basal and the insulin-stimulated rate of 2-deoxyglucose uptake in a dose-dependent manner. Germane to this issue is the report that another inhibitor of PI 3-kinase activity, LY294002, has been shown to decrease both basal and insulin-stimulated glucose transport in 3T3-L1 cells [5,36]. These observations raised the question of the mechanism by which basal glucose transport activity was altered in myoblast cells in response to wortmannin. Based on the fact that GLUT1 is the predominant glucose carrier expressed in L6E9 and Sol8 myoblasts, we focused on the effect of wortmannin on the cell distribution of GLUT1. In keeping with its effects on glucose transport activity, wortmannin caused a dramatic alteration in the distribution of GLUT1 in L6E9 and Sol8 myoblasts, in the absence or presence of insulin. Thus, whereas under basal conditions GLUT1 shows a pattern of distribution characteristic of a cell-surface protein marker, in the presence of wortmannin most GLUT1 was redistributed to an intracellular perinuclear compartment. Wortmannin caused no detectable effect on the distribution of two cell-surface components such as β_1 integrin and the α_1 subunit of the $\text{Na}^+\text{-K}^+\text{-ATPase}$. In this regard, the identification of the specific intracellular compartment(s) that contains most of intracellular GLUT1 in the presence of wortmannin deserves further study. In any case, our data strongly suggest that, in muscle cells, PI 3-kinase is already active under basal conditions (in the absence of insulin) and plays an essential role in the intracellular trafficking of GLUT1, and therefore in the basal rate of glucose transport.

Results shown in Figure 2 clearly demonstrate that insulin stimulates glucose transport in L6E9 and Sol8 myoblasts. Based on the fact that the major glucose transporter expressed in myoblasts is GLUT1, the effect of insulin activating glucose transport must be due to translocation of GLUT1 to the cell surface and/or activation of the carriers already present at the plasma membrane. In this regard, insulin-induced translocation of GLUT1 to the plasma membrane has been reported in

adipocytes [3,37,38]. Immunofluorescence analysis performed in this study did not substantiate the existence of GLUT1 translocation in L6E9 and Sol8 myoblasts. However, this is not definitive evidence against translocation, since immunofluorescence assays might not allow detection of a redistribution of carriers, leading to a 63 % activation of transport as is observed in myoblasts.

PI 3-kinase activity has been implicated in protein-sorting processes. Thus it has been reported that mutant platelet-derived growth factor receptors specifically lacking the binding site for PI 3-kinase fail to accumulate intracellularly [39] and that mutant colony-stimulating factor receptors that do not associate with PI 3-kinase are internalized, but are not delivered to the lysosome for degradation [40,41]. In yeast, the Vps34p PI 3-kinase activity has been shown to be essential for vacuolar protein sorting [42]. Recent data from Stack et al. [43] have revealed important aspects of the Vps34p PI 3-kinase activation pathway. They have shown that an intact Vps15 protein kinase is necessary for the association with and activation of Vps34p. In fact, a functional Vps15-Vps34 complex is absolutely required for the efficient delivery of proteins to the yeast vacuole. In Vps34 mutants lacking PI 3-kinase activity, a dramatic decrease in cellular PI(3)P levels, together with a blockade of vacuolar protein sorting, is observed. In this context, it has been proposed that the phosphorylation of PI by Vps34p may have the effect of physically stimulating membrane curvature and thus facilitating the formation of transport vesicles [44] and/or may recruit or activate proteins involved in the budding or transport of vesicles from the sorting compartment [43]. Based on the fact that Vps34p is a PI-specific PI 3-kinase, it has been suggested that the production of PI(3)P is specifically involved in regulating intracellular protein-sorting pathways [22]. Whether PI(3)P acts in mammalian cells as a second messenger to signal intracellular protein trafficking in a manner similar to that in yeast remains to be determined.

Further studies are required to identify the myoblast isoform(s) of PI 3-kinase which, when inhibited by wortmannin, caused an alteration of GLUT1 cellular distribution and an inhibition of glucose transport, under both basal and insulin-stimulated conditions. Some aspects of our results allow us to put forward the idea that the effect of wortmannin on glucose transport and GLUT1 redistribution might be related to a myoblast functional homologue of yeast Vps34p and/or bovine PI-specific PI 3-kinase. This is based on the following observations. (i) Mammalian PI-specific PI 3-kinase activity was shown to be insensitive to phosphotyrosine peptides from platelet-derived growth factor receptor or insulin receptor substrate-I [21]. This correlates with the fact that the effect of wortmannin on GLUT1 cellular distribution was independent of the presence of insulin. (ii) Similarly to the severe defects in vacuolar protein sorting of yeast strains in which the VPS34 gene had been deleted or those carrying Vps34 point mutations [41,42], in myoblasts wortmannin-induced PI 3-kinase inhibition blocked the cellular trafficking of GLUT1.

In conclusion, results presented here, together with the data discussed above, lead to the concept that the regulation of membrane trafficking may be a function of PI 3-kinases in eukaryotic cells. The biochemical characterization of myoblast PI 3-kinase isoforms and products, together with the possible role of a PI 3-kinase activity in the trafficking of other cellular components, is currently under study in our laboratory.

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