Phosphatidylinositol 3-kinase acts at an intracellular membrane site to enhance GLUT4 exocytosis in 3T3-L1 cells

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Glucose transporters (GLUTs) are continuously recycled in 3T3-L1 cells and so insulin, through its action on phosphatidylinositol 3-kinase (PI 3-kinase), could potentially alter the distribution of these transporters by enhancing retention in the plasma membrane or acting intracellularly to increase exocytosis, either by stimulating a budding or a docking and fusion process. To examine the site of involvement of PI 3-kinase in the glucose transporter recycling pathway, we have determined the kinetics of recycling under conditions in which the PI 3-kinase activity is inhibited by wortmannin. Wortmannin addition to fully insulin-stimulated cells induces a net reduction of glucose transport activity with a time course that is consistent with a major effect on the return of internalized transporters to the plasma membrane. The exocytosis of GLUT1 and GLUT4 is reduced to very low levels in wortmannin-treated cells (≈ 0.009 min^{-1}), but the endocytosis of these isoforms is not markedly perturbed and the rate constants are approx. 10-fold higher than for exocytosis (0.099 and 0.165 min^{-1}, respectively). The slow reduction in basal activity following treatment with wortmannin is consistent with a wortmannin effect on constitutive recycling as well as insulin-regulated exocytosis. PI 3-kinase activity that is precipitated by anti-phosphotyrosine, anti-[insulin receptor substrate 1 (IRS1)] and anti-α-p85 antibodies show the same level of insulin-stimulated activity, ≈ 0.5 pmol/20 min per dish of 3T3-L1 cells. Since the activities precipitated by all three antibodies are similar, it seems unlikely that a second insulin receptor substrate, IRS2, contributes significantly to the insulin signalling observed in 3T3-L1 cells. To examine whether insulin targets PI 3-kinase to intracellular membranes we have carried out subcellular fractionation studies. These suggest that nearly all the insulin-stimulated PI 3-kinase activity is located on intracellular, low-density, membranes. In addition, the association of PI 3-kinase with IRS1 appears to partially deplete the cytoplasm of α-p85-precipitatable activity, suggesting that IRS1 may redistribute PI 3-kinase from the cytoplasm to the low-density microsome membranes. Taken together, the trafficking kinetic and PI 3-kinase distribution studies suggest an intracellular membrane site of action of the enzyme in enhancing glucose transporter exocytosis.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI 3-kinase) is a heterodimeric protein consisting of a catalytic 110 kDa subunit and an α-85 kDa subunit that is necessary for docking to receptor and signalling phosphotyrosine motifs [1,2]. Studies on PI 3-kinase from 3T3-L1 cells have suggested that the catalytic activity of this protein is activated by docking to tyrosine-phosphorylated insulin receptor substrate 1 (IRS1) [3]. In addition to its association with receptors that mediate their effects via tyrosine kinase activity, PI 3-kinases are also implicated in facilitating membrane trafficking processes. The pinocytic activity, membrane ruffling and actin reorganization response observed when KB cells are exposed to insulin has been shown to be due to PI 3-kinase activation [4,5]. PI 3-kinase has been implicated as a key intermediate in the cytoskeletal rearrangements that accompany secretory processes in platelets, basophil and neutrophil cells [6,7]. Similarly, the yeast homologue VPS34 has been shown to be involved in vesicle trafficking and protein sorting [8]. PI 3-kinase is thus an attractive candidate protein as a mediator of insulin action on glucose transport as it could act as a point of convergence of signalling and trafficking processes.

Several recent reports suggest that PI 3-kinase is a crucial signalling intermediate between the insulin receptor and the stimulation of glucose transporter translocation. Evidence that suggests that IRS1-coupled PI 3-kinase activity is important has been obtained using Chinese hamster ovary (CHO) cells transfected with the insulin receptor plus an α-p85 construct in which the p110-binding domain is deleted [9]. The presence of this construct prevents normal association of IRS1 with PI 3-kinase with a consequent disruption of 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. Similarly, the microinjection of this construct into 3T3-L1 cells disrupts the translocation of GLUT4 [10].

An alternative approach, that also implicates PI 3-kinase as a signalling intermediate which is critical for insulin-mediated stimulation of glucose transport, has involved the use of inhibitors which inhibit the catalytic activity of the kinase. These include the fungal metabolite, wortmannin [11–13] and the benzopyran-

Abbreviations used: PI 3-kinase, phosphatidylinositol 3-kinase; GLUT, glucose transporter isoform: IRS, insulin receptor substrate; ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine; CHO, Chinese hamster ovary; $C_{12}E_9$, nonaethyleneglycol-dodecyl ether; DMEM, Dulbecco's modified Eagle's medium; KRH, Krebs–Ringer–Hepes. We use the term exocytosis here to mean the movement of glucose transporter vesicles from intracellular to plasma membranes. This is an externalization event but only the vesicle interior contents are lost to the external medium.

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4-one compound, LY294002 [14]. A strong correlation has been shown between the ability of wortmannin to inhibit PI 3-kinase and insulin-stimulated glucose transport activity; both are inhibited by wortmannin in the nanomolar concentration range ($IC_{50} \approx 5$ nM) in 3T3-L1 cells [13]. Cheatham et al. have shown similar results using LY294002 [14]. Ebina's group have shown that wortmannin inhibits both PI 3-kinase activity and GLUT4 translocation in transfected CHO cells [11] while Okada et al. [12] have shown wortmannin inhibition of glucose transport in rat adipocytes.

Recent studies have begun to address the issue of the site of action of PI 3-kinase in stimulating GLUT4 translocation. Kelly and Ruderman [15] have shown that IRS1-associated PI 3-kinase is mainly associated with intracellular low-density microsomes isolated from rat adipocytes. Interestingly, Kublaoui et al. [16] have shown that there is a strong correlation between insulin action and the endocytosis of autophosphorylated insulin receptor, suggesting an intracellular membrane site of coupling to IRS1. The studies on PI 3-kinase that indicate that it may function in vesicle trafficking suggest that this protein may act quite directly in the trafficking of GLUT4. Therefore, some further definition of the site of action of PI 3-kinase in the GLUT4 recycling pathway may help define more precisely how it mediates its stimulation. Photoaffinity-tagged GLUT4 has been shown to be continuously recycled both in the presence and absence of insulin [17–19]. Kinetic studies using a bis-mannose photolabel have suggested that insulin's major effect on GLUT4 trafficking is to stimulate the exocytosis limb of the recycling pathway [18,20]. PI 3-kinase activation may enhance exocytosis by increasing the budding of GLUT4 from an intracellularly located tubulo-vesicular system or facilitate the movement or docking of vesicles with the plasma membrane. In addition, Czech and Buxton [21] and Jhun et al. [19] have suggested that insulin produces a marked (50–65 %) inhibition of GLUT4 endocytosis. Our results [18,20] suggest a smaller 30 % inhibition. If an insulin-inhibition of endocytosis occurs then insulin activation of PI 3-kinase would be expected to enhance the retention of glucose transporters in the plasma membrane.

GLUT4 translocation is acutely sensitive to insulin which causes very large, 10–20-fold, increases in cell-surface transporter. However, insulin also stimulates the translocation of GLUT1 [20,22] and transferrin receptors [23] in 3T3-L1 cells by increasing exocytosis by $\approx$ 3-fold. It seems that GLUT4 may respond more acutely because it is partially sequestered into a separate vesicular system and away from the constitutively recycling endosome pool that is considered to be responsible for trafficking of GLUT1 and transferrin receptors [23,24]. The pathway by which GLUT1 is internalized is of some significance in 3T3-L1 cells since it is essential that these cells efficiently remove cell-surface GLUT1 in order to maintain very low rates of basal glucose uptake even though total cellular levels of GLUT1 are at least as high [25], if not higher [26], than GLUT4. The contrasting view is that cell-surface GLUT1 is intrinsically inactive in the basal state [27].

To more closely define the site of PI 3-kinase action on regulated and constitutive recycling, we have examined the effects of wortmannin on GLUT1 and GLUT4 recycling kinetics. The data suggest that insulin stimulates glucose transporter exocytosis by stimulating the PI 3-kinase activity at an intracellular site of action. In addition, we have compared the location of IRS1-associated PI 3-kinase with the distribution of α-p85-associated activity. These comparisons suggest that the association of IRS1 with PI 3-kinase, in addition to its role in activating the enzyme, may target the PI 3-kinase to the intracellular low-density fraction of membranes in 3T3-L1 cells.

EXPERIMENTAL

Materials

2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-[^{3}H]bis(D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA) (specific radioactivity $\approx$ 10 Ci/mmol) was prepared as described previously [28], [γ-^{32}P]ATP was from Amersham International. Phosphatidylinositol was from Avanti Polar Lipids (AL, U.S.A.). Dulbecco' modified Eagle's medium (DMEM) was from Flow Laboratories and foetal-bovine serum from Gibco. Monocomponent insulin was a gift from Dr. Ronald Chance, Eli Lilly Corp. Dexamethasone, isobutylmethylxanthine, Protein G–Sepharose, and monoclonal anti-phosphotyrosine–agarose were from Sigma. Streptolysin O was from Murex Diagnostics. Nonaethyleneglycol-dodecyl ether ($C_{12}E_9$; 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 [17,30]. 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 incubation with or without 100 nM insulin for 30 min at 37 °C.

Subcellular fractionation

The method was adapted from that described by Weiland et al. [29]. Material from three 35-mm-diam. plates of 3T3-L1 cells was suspended in 3 ml of TES buffer (10 mM Tris/HCl, 5 mM EDTA, 250 mM sucrose, pH 7.2) and homogenized by 15 strokes in a 5 ml Potter tissue grinder. The homogenate was centrifuged for 15 min at 12500 $\boldsymbol{g}_{\text{max.}}$ to obtain a crude plasma membrane pellet. The supernatant was centrifuged for 15 min at 16000 $\boldsymbol{g}_{\text{max.}}$ to obtain the high-density microsome pellet, followed by 17 min at 554000 $\boldsymbol{g}_{\text{max.}}$ to obtain the low-density microsome pellet. The crude plasma membrane pellet was purified by resuspending it in 1.2 ml of TES buffer followed by centrifugation for 20 min at 104000 $\boldsymbol{g}_{\text{max.}}$ on a 38 % sucrose cushion (1.12 M sucrose, 10 mM Tris/HCl, 5 mM EDTA, pH 7.2). Material suspended on the cushion was pelleted by centrifugation at 76000 $\boldsymbol{g}_{\text{max.}}$. The pelleted material was resuspended in 3 ml of TES and recentrifuged for 9 min at 37000 $\boldsymbol{g}_{\text{max.}}$.

Immunoprecipitation and assay of PI 3-kinase

3T3-L1 cells in 35-mm-diam. dishes or membrane fractions were solubilized in 5 mM Na_2HPO_4, pH 7.2, 0.4 mM sodium orthovanadate, 1 % $C_{12}E_9$, containing 1 μg/ml of the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin and 1 mM dithiothreitol for 20 min, and centrifuged for 20 min at 20000 $\boldsymbol{g}_{\text{max.}}$. Samples (200 μl) of anti-IRS1 (1D6, [9]) or anti-α-p85 monoclonal antibodies (F12, [9]) were preadsorbed on to Protein G–Sepharose (30 μl) for 3 h at 4 °C. The Protein G–Sepharose conjugates and anti-phosphotyrosine–agarose conjugate (25 μl) were washed three times with 5 mM Na_2PO_4, pH 7.2. The sample for immunoprecipitation was 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 % $C_{12}E_9$/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 M NaCl/1 mM dithiothreitol.

The PI 3-kinase activity was measured directly by suspending the immunoprecipitates in 50 μl of buffer containing 20 mM Hepes, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$, 0.2 mg/ml phosphatidylinositol and 40 μM [γ-^{32}P]ATP (0.1 mCi/ml). The phosphatidylinositol was incubated with the immunoprecipitate for 5 min at room temperature and then the MgATP 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 then 20 μl of the lower phase was spotted on to a Silica Gel 60 plate that had been pretreated with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid. The lipids were separated by TLC in the presence of boric acid as described by Walsh et al. [31]. The TLC plates were dried and visualized by autoradiography.

Glucose transport activity

The 3T3-L1 cells were 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 [14].

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 then incubated with wortmannin at the concentrations and for the times indicated in the Figure legends. The dishes were then rapidly washed in KRH buffer at 18 °C and 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. The irradiated cells were washed four times in KRH buffer and solubilized in 1.5 ml of detergent buffer containing 2 % $C_{12}E_9$, 5 mM sodium phosphate and 5 mM EDTA, pH 7.2, and with the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin each at 1 μg/ml. After centrifugation at 20000 $\boldsymbol{g}_{max.}$ 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 [32]. After incubation for 2 h at 0–4 °C, the immunoprecipitates were washed three times with 1.0 % and then once in 0.1 % $C_{12}E_9$ detergent buffer. Labelled glucose transporters were then released from the antibody complexes with 10 % (w/v) SDS, 6 M urea and 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 [32].

Kinetic analysis of glucose transporter trafficking

Time-course data for the net loss of transport activity and glucose transporters were fitted, by non-linear regression, using relative weighting, to eqn. (1)

$$\mathrm{Tp} = \frac{k_{ex}\{1-\exp[-t(k_{ex}+k_{en})]\}}{k_{ex}+k_{en}} + \mathrm{Tp}_0 \cdot \exp[-t(k_{ex}+k_{en})] \qquad (1)$$

where Tp and Tp_0 are the surface transporter at times t and zero, and k_{ex} and k_{en} are the exocytosis and endocytosis rate constants respectively.

RESULTS

The effects of wortmannin on glucose transporter recycling kinetics

When wortmannin addition to 3T3-L1 cells immediately precedes the addition of insulin then, within less than 2 min, it completely inhibits the insulin stimulation of glucose transport activity [10]. By contrast, if cells are initially stimulated by insulin resulting in maximal glucose transport activity and then wortmannin is added, there is a gradual reduction in glucose transport activity. We interpret this reduction in transport activity as being a consequence of the net reduction of transporters at the plasma membrane. Western blotting of samples treated with wortmannin for 30 min confirmed the loss of surface transporters. This net reduction in transporters may occur either because wortmannin stimulates their endocytosis or inhibits their recycling. To analyse this redistribution we have carried out curve-fitting using an

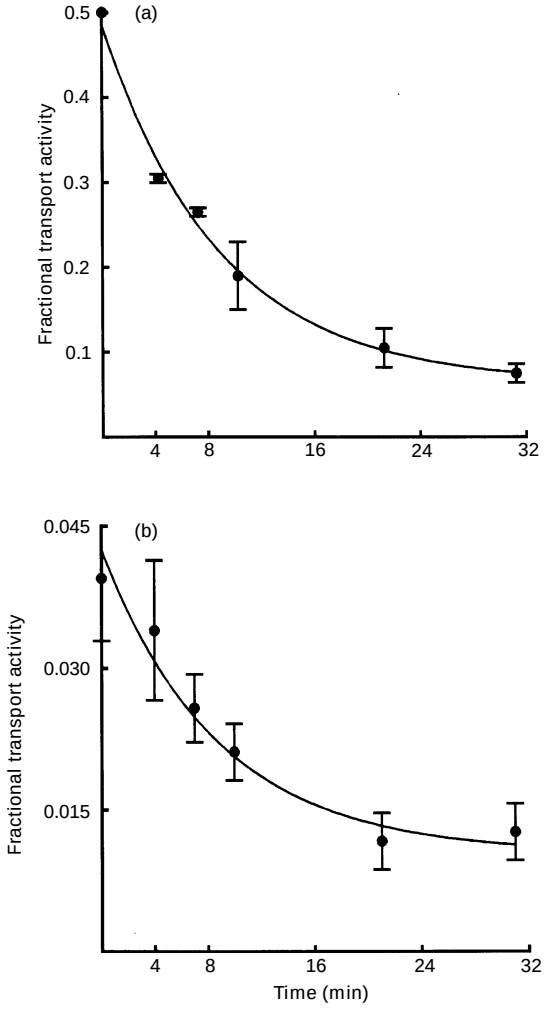

Figure 1 Effect of wortmannin on glucose transport activity in 3T3-L1 cells

Differentiated cells in 35-mm-diam. dishes were treated with (**a**) and without (**b**) 100 nM insulin for 30 min at 37 °C. Wortmannin was added to give a final concentration of 1 μM and the uptake of 0.1 mM 2-deoxy-D-glucose was determined at the indicated times. The transport activity is calculated as a fraction of the initial value in the presence of insulin where half the cell transporters are assumed to be at the cell surface. Results are the mean ± S.E.M. from three experiments (**a**) and four experiments (**b**).

Table 1 Kinetics of glucose transporter trafficking in 3T3-L1 cells

Curve-fitting to eqn. (1) was carried out for the indicated number of experiments and parameters are the mean ± S.E.M.

Assay	Conditions	k_{en} (min^{-1})	k_{ex} (min^{-1})	Tp_o (min^{-1})
Glucose transport activity	Insulin + wortmannin ($n = 3$)	0.115 ± 0.031	0.007 ± 0.002	0.49 ± 0.03
	Basal + wortmannin ($n = 4$)	0.120 ± 0.017	0.002 ± 0.002	0.043 ± 0.007
Reduction in cell-surface transporters	GLUT1 + wortmannin ($n = 4$)	0.099 ± 0.013	0.009 ± 0.002	0.520 ± 0.018
	GLUT4 + wortmannin ($n = 4$)	0.165 ± 0.007	0.009 ± 0.002	0.530 ± 0.017
Steady-state recycling*	Insulin GLUT1	0.093 ± 0.017	0.096 ± 0.023	
	Insulin GLUT4	0.080 ± 0.007	0.086 ± 0.011	
	Basal GLUT1	0.121 ± 0.020	0.035 ± 0.009	
	Basal GLUT4	0.116 ± 0.006	0.010 ± 0.001	

* From [20].

equation that gives an estimate of the endocytosis and exocytosis rate constants (see the Experimental section). In applying this equation to analysis of transport data a number of simplifications are necessary. First, we do not distinguish between the behaviour of GLUT4 and GLUT1 although photolabelling experiments described below give information on the separate recycling behaviour. Secondly, we assume that half the cellular transporters are at the plasma membrane at the beginning of the time-course experiment. Previous studies have suggested that this is a reasonable approximation [25] and recalculation of the results using a different starting point does not markedly alter the calculated values for the rate constants of endocytosis.

Figure 1(a) shows that the decline in transport activity from the maximally insulin-stimulated level occurs with a half-time of 5.7 min corresponding with a rate constant of 0.122 min^{-1}, which represents the sum of the exocytosis and endocytosis rate constants (eqn. 1). This half-time is similar to that previously observed for the decrease in transport activity following the removal of insulin by a low-pH wash or by inhibiting insulin action on GLUT4 translocation with phenylarsine oxide [17]. When analysed to estimate the recycling rate constants the data show that the half time is mainly determined by an endocytosis rate constant, $k_{en.} = 0.115\ min^{-1}$, and a slow exocytosis rate constant, $k_{ex.} = 0.007\ min^{-1}$ (Table 1).

To determine the separate rates of loss of GLUT1 and GLUT4 from the cell surface following wortmannin treatment of insulin-stimulated cells, we have used the bis-mannose photolabel ATB-BMPA. This reagent is impermeable and will only react with those glucose transporters that remain exposed at the cell surface. Following labelling at the indicated times (Figure 2), GLUT1 and GLUT4 are immunoprecipitated. The decline in surface available transporters is plotted as a fraction of those transporters present at the cell surface in fully insulin-stimulated cells, that is, 50 % of the total ([25] and confirmed in the present study). Both isoforms are extensively internalized following wortmannin treatment but GLUT4 declines more rapidly than GLUT1; the half-times are 4.2 and 6.4 min respectively. Further analysis of these time courses shows that the exocytosis of both GLUT4 and GLUT1 are reduced to very low values. Comparing these values with those occurring in fully insulin-stimulated cells (Table 1) suggests that the predominant effect of wortmannin is to reduce exocytosis of both isoforms, to 0.009 min^{-1}, which is less than 10 % of the insulin-stimulated level. In basal cells, the exocytosis of GLUT1 is faster than that of GLUT4, that is, GLUT1 is less efficiently retained intracellularly [20]. However, in wortmannin-treated cells (Figure 2) the rates of GLUT4 and GLUT1 exocytosis are both reduced to very low levels and the difference

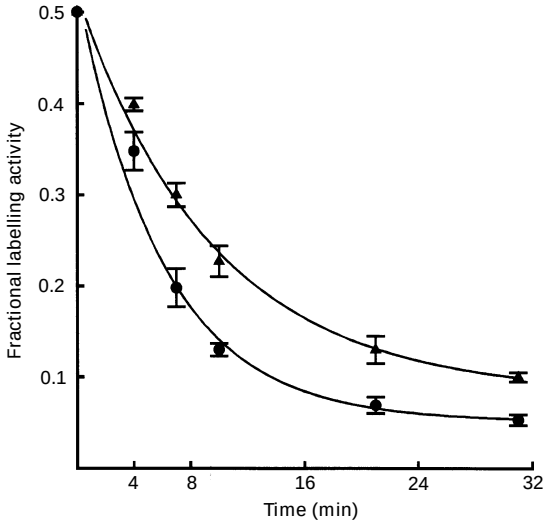

Figure 2 Effect of wortmannin on cell-surface GLUT4 and GLUT1

Differentiated 3T3-L1 cells in 35-mm-diam. dishes were treated with 100 nM insulin for 30 min at 37 °C. Wortmannin was added to give a final concentration of 0.1 μM. At the indicated times the dishes were rapidly washed with KRH buffer at 18 °C and the net loss of cell-surface GLUT1 (▲) and GLUT4 (●) were determined by photolabelling for 1 min with ATB-[2-^{3}H]BMPA at 18 °C. GLUT1 and GLUT4 were then immunoprecipitated and resolved on SDS/PAGE gels. The labelling activity is calculated as a fraction of the initial value in the presence of insulin where half the cell transporters are assumed to be at the cell surface. Results are the mean ± S.E.M. from four experiments.

in exocytosis of the two isoforms is eliminated. In the wortmannin-treated cells a small but reproducible difference between the endocytosis rate constants for the two isoforms is evident. The endocytosis rate constants of GLUT4 and GLUT1 are 0.165 and 0.099 min^{-1} respectively (Table 1).

If glucose transport activity is measured in basal cells immediately following wortmannin addition then there is an insignificant reduction in transport activity [13]. However, Figure 1(b) shows that if wortmannin is added to the 3T3-L1 cells and transport activity is measured at a series of time-points up to 30 min then there is a gradual decline in transport activity with a half-time of 5.7 min corresponding with a rate constant of 0.122 min^{-1}. To analyse the data further, we have taken the relative transport rates in basal and insulin-stimulated cells as an

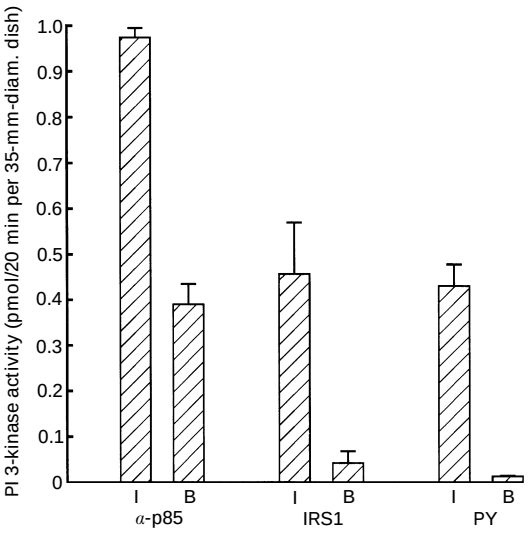

Figure 3 Immunoprecipitation of PI 3-kinase activity from detergent lysates of 3T3-L1 cells

3T3-L1 cells in 35-mm-diam. dishes were treated with (I) and without (B) 100 nM insulin for 30 min at 37 °C. The cells were then solubilized in buffered $C_{12}E_9$ detergent solution and PI 3-kinase activity was precipitated with either anti-α-p85 and anti-IRS1 antibodies preadsorbed on to Protein G–Sepharose or anti-phosphotyrosine antibody covalently coupled to agarose. PI 3-kinase activity was determined by incubation with ATP and phosphatidylinositol followed by separation of labelled phospholipids by TLC in a borate solvent system. This separates phosphatidylinositol 3-phosphate from phosphatidylinositol 4-phosphate. The radioactivity corresponding to phosphatidylinositol 3-phosphate was determined by cutting and counting. Results are the mean ± S.E.M. from four experiments.

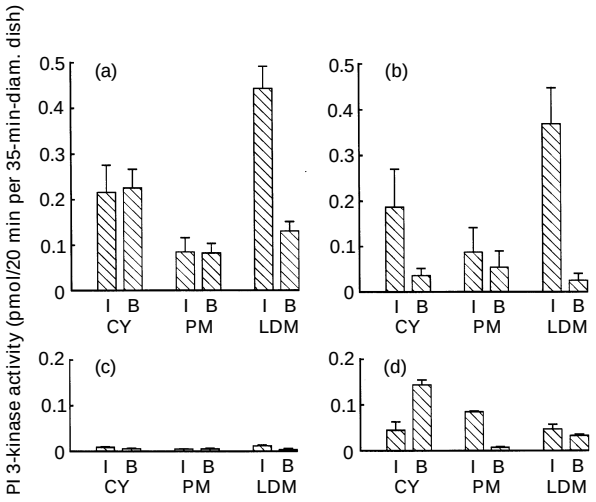

Figure 4 Immunoprecipitation of PI 3-kinase activity from subcellular membrane fractions of 3T3-L1 cells

Monoclonal antibodies to α-p85 and IRS1, preadsorbed on to Protein G, were added to membrane fractions solubilized in $C_{12}E_9$ detergent buffer. PI 3-kinase activity was determined as described in the legend to Figure 3. First immunoprecipitations were with either anti-α-p85 antibody (**a**) or anti-IRS1 antibody (**b**). The supernatants (**a**) were subjected to second precipitation with anti-IRS1 antibody (**c**) while the supernatants (**b**) were subjected to a second precipitation with anti-α-p85 antibody (**d**). Results are the mean ± S.E.M. from 4–9 experiments.

indication of the fraction of transporters present at the cell surface at the beginning of the time course. The calculated endocytosis and exocytosis rate constants are then 0.120 and 0.002 min^{-1} respectively. The net reduction in transport activity therefore appears to be a consequence of inhibition of exocytosis by wortmannin. This is a somewhat surprising result which suggests that wortmannin may inhibit constitutive exocytosis of glucose transporters as well as inhibiting insulin-regulated exocytosis.

The association of PI 3-kinase activity with IRS1 and α-p85

To estimate the proportion of PI 3-kinase that is associated with IRS1 in 3T3-L1 cells, we have immunoprecipitated PI 3-kinase activity with three different antibodies, to phosphotyrosine, IRS1 and α-p85 (Figure 3). The insulin-stimulated increase in PI 3-kinase activity associated with all three antibodies is similar, $\approx$ 0.5 pmol/20 min per 35-mm-diam. dish. Use of both the anti-IRS1 and the anti-phosphotyrosine antibodies produces large, $\approx$ 10-fold, relative changes comparing basal and insulin-stimulated cells, while the anti-α-p85 antibody only produces a 2-fold change following insulin stimulation of the cells. Therefore, in basal cells a large proportion of PI 3-kinase is associated with α-p85 but not with IRS1.

To analyse further the differences in distribution of IRS1- and α-p85-precipitatable activity we have compared the activity in plasma membrane, low-density microsome and cytoplasmic fractions (Figure 4). The amounts of PI 3-kinase activity recovered in the high-density microsome fraction were very low and were not routinely analysed. The amount of α-p85-precipitatable activity is greatest in the low-density microsome fraction and this is the main location of the insulin-stimulated activity. From examination of data from whole-cell lysates (Figure 3), it is evident that there is a large amount of PI 3-kinase activity associated with the α-p85 precipitates but not associated with the insulin response. This additional activity is located in the cytoplasmic fraction. The lowest levels of PI 3-kinase activity are associated with the plasma membrane fraction.

The IRS1 precipitation results show a lower amount of PI 3-kinase in the cytoplasmic fraction than in the α-p85 precipitates. The highest levels of IRS1-associated PI 3-kinase are in the low-density microsome fraction. The level of insulin stimulation of activity in this fraction is equal to that observed when immunoprecipitating from whole-cell detergent lysates (Figure 3). To analyse whether there is residual activity in the supernatants from the anti-IRS1 antibody precipitations, a second precipitation was carried out with the anti-α-p85 antibody. The basal activity is higher than the activity of the insulin-treated samples, suggesting that association of PI 3-kinase with IRS1 depletes the cytoplasm of activity. Very low levels of PI 3-kinase are detected in the low-density microsome fraction with the anti-α-p85 antibody following precipitation by the anti-IRS1 antibody (Figure 4d). This suggests that most, if not all, of the PI 3-kinase activity in the low-density microsome fraction is associated with IRS1. As expected, if the anti-IRS1 antibody is used after the anti-α-p85 antibody then no residual PI 3-kinase is detected. This suggests that all the IRS1-associated PI 3-kinase activity is associated with α-p85 (Figure 4c).

DISCUSSION

In the absence of insulin, GLUT4 resides in intracellular membranes of the insulin-target tissues of fat and muscle [33–37]. On insulin stimulation there is a redistribution of these transporters so that approximately half of the cellular GLUT4 resides at the plasma membrane [25,38]. This redistribution could occur if

insulin either inhibits the GLUT4 endocytosis process or stimulates the exocytosis. Several approaches have been used in an attempt to resolve this question. Experiments in which we have used the bis-mannose photolabel suggest that by far the major effect of insulin is on GLUT4 exocytosis ($\approx$ 9-fold) with a smaller $\approx 30\,\%$ inhibition of endocytosis [18,20]. A similar approach using a different photolabel suggests that GLUT4 endocytosis is reduced by $\approx 65\,\%$ following insulin treatment [19].

We have taken two approaches here to address the issue of whether the PI 3-kinase-dependent step in insulin action involves an inhibition of the return of transporters from the plasma membrane to the low-density microsomes or a stimulation of translocation from intracellular membranes.

Measurement of glucose transporter recycling kinetics

The kinetic approach described here does not utilize a subcellular fractionation technique. Instead, we simply use the photolabel at different times in a time-course experiment to determine the net loss of GLUT1 and GLUT4 that occurs following a wortmannin treatment. Analysis of the rate constants shows that wortmannin produces an approx. 40–50 % increase in GLUT4 endocytosis compared with experiments in which insulin steady-state recycling has been studied (Table 1). However, the main effect of wortmannin is to inhibit the exocytosis of both GLUT1 and GLUT4. The rate constants for exocytosis are $\approx$ 10-fold higher in the insulin steady-state recycling experiments. Since the exocytosis of GLUT1 is normally higher than that of GLUT4 in basal cells (Table 1, [20]), the results suggest that the constitutive exocytosis of GLUT1, as well as the regulated exocytosis of both isoforms, is inhibited. Consistent with a PI 3-kinase involvement in constitutive exocytosis, we find a slow reduction in basal glucose transport following treatment with wortmannin. It is at present unclear whether the constitutive exocytosis of GLUT1 in basal cells is occurring via the same route as GLUT4, but at a faster rate. If the routes are different then this implies that there may be more than one isoform of PI 3-kinase; one being involved in constitutive exocytosis and the other enhancing exocytosis from a separate compartment in response to the production of tyrosine-phosphorylated IRS1. Kinetic analysis of GLUT4 trafficking has previously led us to suggest that trafficking may occur through two consecutive intracellular pools and that a greater proportion of GLUT4 than GLUT1 accesses a specialized pool that only slowly recycles in the basal state [24]. The non-specialized endosome pool may be responsible for the recycling of transferrin and other receptors.

The rate constants of GLUT4 endocytosis that we have measured, both with the approach described here and previously using steady-state trafficking of tracer-tagged transporter (≈ 0.1–$0.15\ \mathrm{min}^{-1}$), are similar to those determined for the endocytosis of transferrin receptor chimeras, with either GLUT4 N- or C-terminal targeting regions in CHO cells [39]. However, the implication from studies of transferrin receptor constructs is that GLUT1 (which contains neither of these targeting regions) should be endocytosed with a rate constant that is similar to that of bulk membrane flow, which in CHO cells is $\approx 0.03\ \mathrm{min}^{-1}$ [39]. Our earlier study [20] showed no significant difference between the endocytosis of GLUT4 and GLUT1 in basal cells. This may have been because we were dealing with very low levels of labelling of these isoforms in the basal state. The problem of resolution has been eliminated in the present study but we still see a rate of internalization of GLUT1 that is only 50 % slower than that of GLUT4 and much faster than would be expected from the data on bulk flow internalization in CHO cells. A similar approach to that described here has been previously taken but using a low pH wash to remove insulin and measure the consequent loss of GLUT4 and GLUT1 from the cell surface [17]. In these studies, the half-times of GLUT4 and GLUT1 internalization were 6.8 and 9.2 min respectively. If we now use the steady-state values for the distribution of these isoforms [25] then the calculated rate constants for GLUT4 and GLUT1 endocytosis are 0.10 and $0.068\ \mathrm{min}^{-1}$ respectively. Although these values are slightly slower than those now estimated using wortmannin, both studies suggest that there is only an $\approx 50\,\%$ slower endocytosis of GLUT1 than of GLUT4.

There are several possible explanations for the higher than expected endocytosis of GLUT1 in 3T3-L1 cells in the apparent absence of strong internalization motifs of the types described for GLUT4 [40–42]. First, GLUT1 may possess a so far unrecognized targeting motif. Secondly, 3T3-L1 cells may possess internalization pathways that are independent of clathrin-coated vesicles and which may use an association with caveolin to internalize cell-surface proteins [43]. Indeed, it has been recently suggested that an $\approx 10\,\%$ portion of 3T3-L1 cell GLUT4 becomes associated with caveolin during insulin stimulation of translocation [43]. Thirdly, the relatively fast GLUT1 endocytosis may occur because bulk membrane flow is faster in 3T3-L1 cells than in CHO cells. Studies measuring the endocytosis of sucrose as a fluid marker suggest that there is a relatively fast equilibration with an endosome compartment over 10–15 min followed by transit of sucrose to a second compartment over several hours [44]. Using lucifer yellow we have shown that this marker of pinocytic activity equilibrates with 3T3-L1 cells with a half-time of $\approx$ 7 min (J. Yang, and G. D. Holman, unpublished work).

Activity and distribution of PI 3-kinase in 3T3-L1 cells

The second approach that we have taken to test the possibility that PI 3-kinase facilitates translocation by acting at an intracellular membrane site is to determine the activities and location of both α-p85- and IRS1-precipitatable PI 3-kinase. Recent studies on transgenic rats in which the IRS1 is deleted have suggested that IRS1 is not essential for insulin stimulation of glucose transport, which is still partially insulin responsive in the absence of IRS1. Studies on these animals suggest that a second insulin receptor substrate molecule IRS2 may take over the signalling role [45,46]. The question then arises as to the relative abundance of IRS1 and IRS2 in normal insulin-responsive cells. Our results suggest that in 3T3-L1 cells, IRS1 may be the major substrate of the insulin receptor that is coupled to PI 3-kinase. If we compare the increments in the PI 3-kinase activity that occur in insulin-stimulated cells (Figure 3) then we see that an anti-α-p85 and an anti-phosphotyrosine antibody produce the same insulin stimulation level as that observed with the anti-IRS1 antibody. If a second IRS was present in significant quantities then the levels of precipitation by the anti-α-p85 and anti-phosphotyrosine antibodies should be greater than we observe. Clearly such considerations depend on the ability of these antibodies to quantitatively precipitate all the activity that is available. We have tried increasing the amounts of these antibodies but do not observe any additional precipitation. Furthermore, it seems unlikely that all three antibodies would give the same level of insulin-stimulated activity if one of the antibodies gave anomalously low precipitation efficiency.

The fractionation data show that, as in rat adipocytes [15], most of the IRS1-associated PI 3-kinase activity is located in the low-density microsomes. Following IRS1 precipitation there is a partial depletion of α-p85-precipitatable activity from the cytoplasm (Figure 4d). The association of IRS1 with the PI 3-kinase

heterodimer increases the total cell lysate activity that is precipitatable by the anti-α-p85 antibody (Figure 3). This change in activity [3] tends to obscure a redistribution of the activity between the cytosolic and low-density microsome fractions. The redistribution is more obvious if the sum of the cytosol and low-density microsome activities are separately normalized to 100% for the basal and insulin-stimulated states. Then, in the basal state 58% of the activity is in the cytosol and 42% on the low-density microsomes. In insulin-treated cells the normalized activity in the cytosol is reduced to 33% with a corresponding increase, to 67%, in the low-density microsome fraction. The coupling with IRS1 is probably responsible for recruiting the activity on to the low-density microsomes.

The study by Kelly and Ruderman [15] suggests that, in rat adipocytes, PI 3-kinase may occur on a membranous system that is lighter than GLUT4 vesicles. Further studies will be needed to further resolve the location of the PI 3-kinase within intracellular membrane systems and the extent of association of PI 3-kinase with GLUT4 vesicles in 3T3-L1 cells. PI 3-kinase may not be tightly associated with GLUT4 vesicles if the enzyme were responsible for a vesicle budding process. Such a process could release vesicles from a preformed tubulo-vesicular system with attached 'lace-like' buds similar to those observed in detailed examination of Golgi morphology by tomographic electron microscopy [47]. Alternatively, PI 3-kinase may be involved in removing some constraint to vesicle translocation such as an association with actin [48,49] or a docking/fusion clamp similar to that implicated in the regulation of synaptic vesicle trafficking [50].

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