Compartment-specific regulation of phosphoinositide 3-kinase by platelet-derived growth factor and insulin in 3T3-L1 adipocytes

Barbara T. NAVÉ, Richard J. HAIGH, Amanda C. HAYWARD, Kenneth SIDDLE and Peter R. SHEPHERD*†
Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR, U.K.

To understand how the stimulation of phosphoinositide 3-kinase (PI 3-kinase) by different growth factors can activate different subsets of downstream responses, growth-factor regulation of PI 3-kinase activity at different intracellular locations was investigated in 3T3-L1 adipocytes. Insulin caused a large stimulation of glucose transport and stimulated recruitment of transferrin receptors to the plasma membrane (PM) in these cells, whereas platelet-derived growth factor (PDGF)-bb was virtually without effect on these responses. Subcellular fractionation studies after stimulation with PDGF-bb or insulin revealed a differential effect of these growth factors on subcellular localization of PI 3-kinase activity. PDGF was more effective than insulin in stimulating PI 3-kinase activity and recruiting the p85α PI 3-kinase adaptor subunit in the fraction containing the PM. However, in the microsomal fraction insulin significantly increased PI 3-kinase activity and p85α levels, whereas PDGF was almost without effect. In the microsomal membrane fraction the insulin-stimulated recruitment of p85α closely matched the increase PI 3-kinase activity, indicating that insulin stimulation of PI 3-kinase in this fraction is largely due to recruitment of PI 3-kinase enzyme rather than alterations in specific activity. Insulin-stimulated recruitment of p85α to the microsomal membranes was not inhibited by wortmannin, indicating that PI 3-kinase activity was not required for this process. A further level of compartment-specific regulation of PI 3-kinase in response to PDGF was revealed by the finding that tyrosine phosphorylation of the p85α adaptor was restricted to the PM-containing fraction. Insulin had no effect on p85 tyrosine phosphorylation in either fraction. In summary, these results suggest a basis by which insulin and PDGF could both use PI 3-kinase signalling cascades but achieve different signalling outcomes.

INTRODUCTION

Recent studies have revealed a number of the intracellular signalling pathways that are activated by growth-factor receptor tyrosine kinases [1,2] and have shown that there is a great deal of overlap in the pathways that are activated by different receptor tyrosine kinases. An example of this is the enzyme phosphoinositide 3-kinase (PI 3-kinase), which is acutely activated by a number of growth factors [3]. A key role for PI 3-kinase in growth-factor signalling is indicated by recent studies using expression of mutants with decreased PI 3-kinase activity [4] or using the selective PI 3-kinase inhibitors wortmannin [5] and LY294002 [6]. These studies suggest that PI 3-kinase is essential for growth-factor stimulation of a wide range of responses including membrane ruffling [7], stimulation of glucose transporter and transferrin receptor translocation [4,8–11], activation of glycogen synthase [12], inhibition of lipolysis [9], stimulation of the mitogen-activated protein kinase cascade [13], inhibition of glycogen synthase kinase 3 [13], activation of $p70^{s6}$ kinase [11], activation of the small GTP-binding protein Rac [14] and activation of protein kinase-B/akt [15].

However, although many growth factors activate PI 3-kinase, each of these growth factors only activates a subset of the PI 3-kinase-dependent pathways described above. For example, although both insulin and platelet-derived growth factor (PDGF) stimulate PI 3-kinase, only insulin significantly stimulates glucose transport in 3T3-L1 adipocytes [16]. Although this could indicate that activation of PI 3-kinase activity alone is not sufficient to induce these responses, the possibility also exists that individual growth factors differentially regulate PI 3-kinase activity at specific cellular locations and that this is responsible for differences in their effects on cellular responses. Therefore we have investigated the effects of insulin and PDGF on PI 3-kinase activity at specific subcellular locations in 3T3-L1 adipocytes and correlated this with the ability of these growth factors to induce increased glucose transport and recruitment of transferrin receptors. These studies demonstrate that PDGF and insulin differentially regulate PI 3-kinase at specific intracellular sites in 3T3-L1 adipocytes by inducing differential recruitment and/or tyrosine phosphorylation of the PI 3-kinase enzyme at these locations.

MATERIALS AND METHODS

Materials

^{125}I-labelled Protein A was obtained from New England Nuclear; all other radiochemicals and enhanced chemiluminescence reagents were from Amersham; wortmannin, phosphatidylinositol, human holo-transferrin (receptor grade) and insulin were from Sigma; PDGF-bb, PDGF-aa and basic fibroblast growth factor (b-FGF) were from Gibco; phosphocreatine and creatine kinase were from Boehringer; horseradish peroxidase-linked transferrin was from Jackson; PY-20 anti-phosphotyrosine antibody was from ICN-Flow; 4G-10 anti-phosphotyrosine antibody was from UBI. Rabbit polyclonal antibodies to the PI 3-kinase p85α

Abbreviations used: KRP, Krebs–Ringer phosphate; PDGF, platelet-derived growth factor; b-FGF, basic fibroblast growth factor; IRS-1, insulin receptor substrate 1; NP40, Nonidet P40; PI 3-kinase, phosphoinositide 3-kinase; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PM, plasma membrane; TGN, trans-Golgi network.

* Present address: Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.
† To whom correspondence should be addressed.

subunit were raised by using glutathione S-transferase fusion proteins containing the N-terminal SH2 domain or the SH3/BCR domains of the human p85 subunit of PI 3-kinase (using cDNA supplied by Dr. J. Schlessinger). Additional p85α rabbit polyclonal antibodies to the N-terminal SH2 domain were purchased from UBI. Monoclonal antibodies to p85α SH3 domain (U13) and p85β were supplied by Professor M. Waterfield. Polyclonal antibody to the C-terminus of GLUT-4 was a gift from Dr. G. Gould (University of Glasgow, Glasgow, U.K.), and polyclonal antisera to the *trans*-Golgi network marker TGN38 and mannose 6-phosphate receptor were provided by Dr. P. Luzio (University of Cambridge, Cambridge, U.K.).

Cell culture

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as previously described [12]. Cells were used 7–11 days after differentiation.

Hexose transport

[^{3}H]2-Deoxyglucose uptake was determined as previously described [17]. Cells were incubated in serum-free Dulbecco's minimal essential medium for 3 h and then transferred to Krebs–Ringer phosphate (KRP) buffer, pH 7.4.

Transferrin receptor recruitment

Cells were stimulated with appropriate growth factor and then washed three times with ice-cold KRP buffer, pH 7.3. Tracer ^{125}I-labelled transferrin was allowed to bind in KRP buffer for 90 min at 4 °C, essentially as previously described [18]. Non-specific binding was determined in the presence of 1 μM holo-transferrin.

Preparation of membrane fractions

3T3-L1 adipocytes were serum-starved overnight and then treated with growth factor. Cells were disrupted by ten passages through a 25-gauge needle in Tris/sucrose buffer (0.25 M sucrose, 20 mM Tris/HCl, pH 7.4, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 200 kallikrein inhibitor units/ml aprotonin, 1 μg/ml antipain, 400 μg/ml benzamidine and 1 μg/ml pepstatin). Nuclei and unbroken cells were removed by centrifugation at 3000 ***g*** for 10 min. Total membrane fractions were prepared by centrifugation of the 3000 ***g*** supernatant for 30 min at 350000 ***g*** with a Beckman TL 100 centrifuge. Fractionation studies were performed on cells disrupted by the same protocol as described above except that the 3000 ***g*** supernatant was centrifuged at 18000 ***g*** for 20 min. The microsomal fraction was collected by centrifugation of the 18000 ***g*** supernatant for 30 min at 350000 ***g*** with a Beckman TL 100 centrifuge. The resultant pellet was referred to as the microsomal fraction, and the resultant supernatant was referred to as the cytosol fraction. All centrifugation steps were performed at 4 °C. All pellets were resuspended in PI 3-kinase assay buffer (see below). To verify the separation of plasma membranes from low-density microsomes, 5′-nucleotidase activity was assayed in all fractions as previously described [19] and membrane fractions were immunoblotted with antibodies to the glucose transporter GLUT4, to TGN38 and to the mannose 6-phosphate receptor.

Immunoprecipitation of phosphotyrosine-containing proteins

3T3-L1 adipocytes were serum-starved overnight and then treated with growth factor for the appropriate time. Cells were washed once with ice-cold PBS then lysed in ice-cold buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM Na_3VO_4, 10 μg/ml leupeptin, 0.2 mM PMSF, 10 % (w/v) glycerol and 1 % (w/v) Nonidet P40 (NP40). Where membrane fractions were used as starting material, the same buffer was used to solubilize membranes. Cleared lysates were immunoprecipitated with PY-20 anti-phosphotyrosine antibody as previously described [20]. To remove any traces of NP40, immunoprecipitates were washed extensively as described [20].

PI 3-kinase activity assay

PI 3-kinase enzymic activity was assayed at 37 °C by measuring the incorporation of ^{32}P from γ-labelled ATP into phosphatidylinositol in buffer at pH 7.4 containing 20 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 30 mM NaCl, 1 mM DTT, 3 mM $MgCl_2$, 1.2μM ATP, 0.6 mg/ml phosphatidylinositol and 0.33 % cholate. Experiments confirmed that production of lipid product was linear for up to 20 min under the conditions used (results not shown). The ^{32}P-labelled phospholipid product was resolved by TLC as described [20], with a single band being observed in reactions from anti-phosphotyrosine immunoprecipitates. This band was not observed when the incubations were performed in the presence of 0.1 % NP40 or 10 nM wortmannin, indicating that the band was the product of PI 3-kinase activity. Bands were quantified with a Fuji Bas 2000 Phosphorimager.

PI kinase activity in membrane fractions

Assays in membrane fractions were performed in essentially the same manner as for immunoprecipitates, except that resuspended membranes containing equal amounts of protein were incubated in place of the immunoprecipitate complex. High levels of ATPase activity in the plasma membrane necessitated the use of an ATP regenerating system (10 m-units/ml creatine kinase and 15 mM phosphocreatine) and a shorter assay incubation time (10 min) to achieve a linear response in the assay.

RESULTS

Effects of growth factors on PI 3-kinase activity, glucose transport and transferrin receptor recruitment

A primary aim of this study was to understand how different growth factors are able to stimulate PI 3-kinase and yet only induce a subset of PI 3-kinase-dependent responses. We investigated the effects of PDGF-bb, b-FGF and insulin on PI 3-kinase activity, glucose transport and transferrin receptor recruitment in 3T3-L1 adipocytes. The concentrations of PDGF-bb, b-FGF and insulin used in the present study were found to give maximal stimulations of DNA synthesis as, measured by thymidine uptake (results not shown), indicating that cognate receptor systems existed for these ligands in this cell type. PI 3-kinase activity was very low in anti-phosphotyrosine immunoprecipitates from serum-starved cells but was increased markedly by insulin and PDGF-bb, although b-FGF had no effect (Table 1). In agreement with previous studies, insulin caused a large stimulation of glucose transport, whereas PDGF-bb and b-FGF caused very small stimulations [16,21] (Table 1). Although insulin has previously been shown to stimulate transferrin receptor recruitment in adipocytes [18], the effects of other growth factors have not been investigated. The present study demonstrates that insulin stimulated transferrin receptor recruitment but b-FGF and PDGF-bb had no effect (Table 1).

Table 1 Effect of growth factors in stimulating PI 3-kinase activity, glucose transport and transferrin receptor recruitment

Serum-starved 3T3-L1 adipocytes were stimulated with insulin (10 nM), PDGF-bb (20 ng/ml) or b-FGF (25 ng/ml) as indicated for 10 min. PI 3-kinase activity was determined in anti-phosphotyrosine immunoprecipitates and [^{3}H]2-deoxyglucose uptake and ^{125}I-labelled transferrin binding to the cells were all determined as described in the Materials and methods section. Data represent means ± S.E.M. for at least three independent experiments.

Stimulus	Anti-phosphotyrosine immunoprecipitable PI 3-kinase activity (fold over basal)	2-Deoxyglucose uptake (fold over basal)	^{125}I-transferrin binding (fold over basal)
Insulin	17.2 ± 4.8	11.4 ± 0.6	1.80 ± 0.09
PDGF-bb	49.9 ± 12.3	2.2 ± 0.1	1.09 ± 0.06
b-FGF	1.9 ± 0.4	2.1 ± 0.1	1.16 ± 0.05

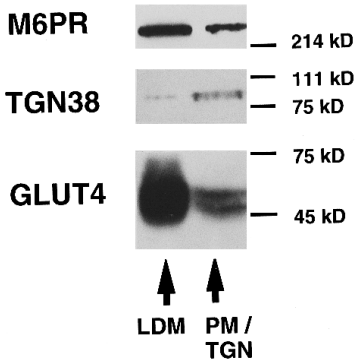

Figure 1 Characterization of subcellular fractions

Membrane fractions were prepared from 3T3-L1 adipocytes as described in the Materials and methods section. The 18000 ***g*** pellet fraction typically contained 3–5-fold more protein than the high-speed pellet, so lanes were loaded to reflect the total amount of each protein in each fraction. Microsomal fraction (LDM; 15 μg of membrane protein) and PM-containing fraction (PM/TGN; 70 μg of membrane protein) were separated by SDS/PAGE and transferred to poly(vinylidene difluoride) filters. Filters were Western blotted with polyclonal antibodies to GLUT4 glucose transporter, the mannose 6-phosphate (IGF2) receptor (M6PR) and TGN38. Bands were revealed by enhanced chemiluminescence. Abbreviation: kD, kDa.

Growth-factor stimulation of PI 3-kinase activity in subcellular fractions

In total membrane fractions prepared from 3T3-L1 adipocytes, insulin and PDGF each stimulated PI 3-kinase 2–3-fold (results not shown). To determine whether this was the case in all membrane fractions within the cell, the effect of these growth factors in stimulating PI 3-kinase activities in subcellular fractions was analysed. The subcellular fractionation technique was designed to separate the plasma membrane (PM) and Golgi network from the microsomal fraction in which the translocatable GLUT4 pool is found. The fractions were biochemically characterized by assaying for the PM-specific marker 5′-nucleotidase [19], and 92–95 % of the total 5′-nucleotidase activity was present in the 18000 ***g*** pellet. Membrane fractions were also immunoblotted for GLUT4, mannose 6-phosphate receptor and the trans-Golgi network (TGN) marker TGN38 (Figure 1). The gel was loaded to reflect the fact that the total amount of protein in the 18000 ***g*** pellet was 3–5-fold more than in the 350000 ***g*** pellet. Most of the TGN38 (Figure 1) was found in the 18000 ***g*** pellet and most of the GLUT4 was present in the 350000 ***g*** pellet,

Table 2 Effects of PDGF-bb and insulin in stimulating PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates from different subcellular membrane fractions

Overnight serum-starved cells were stimulated with PDGF-bb (20 ng/ml) or insulin (10 nM) for 10 min. Subcellular fractions were prepared as described in the Materials and methods section. Anti-phosphotyrosine immunoprecipitates were prepared from these membrane fractions and PI 3-kinase activity was measured as described in the Materials and methods section. Results are expressed as fmol of phosphate transferred per min per 10 cm dish of 3T3-L1 adipocytes. Results are the means ± S.E.M. for three separate experiments.

	PI 3-kinase activity (fmol per min per dish)		
Stimulus	PM-containing fraction	Microsomal membranes	Cytosol
Basal	0.62 ± 0.12	1.06 ± 0.25	0.69 ± 0.19
Insulin	4.58 ± 1.04	4.89 ± 0.43	2.86 ± 0.54
PDGF-bb	23.14 ± 2.26	2.50 ± 0.47	8.27 ± 1.63

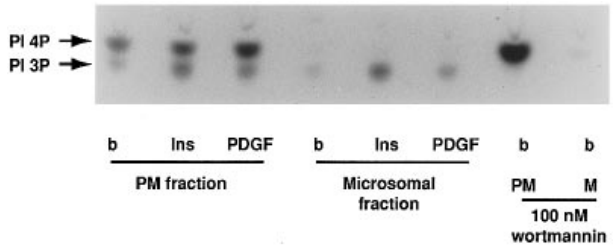

Figure 2 PI 3-kinase activity associated with subcellular membrane fractions

Serum-starved 3T3-L1 adipocytes were incubated with vehicle (lanes marked 'b'), insulin (Ins; 10 nM) or PDGF-bb (20 ng/ml) for 10 min. Membrane fractions were prepared and assayed for PI kinase activity as described in the Materials and methods section. To define which band represented PI 3-kinase activity, the PI kinase assay was also performed in membranes preincubated for 2 min with 1 μM wortmannin. Results show a representative experiment and similar results were obtained in at least three separate experiments.

whereas the mannose 6-phosphate receptor was spread more evenly between fractions. As a result of these studies the 18000 ***g*** fraction was referred to as the PM-containing fraction and the 350000 ***g*** pellet was referred to as the microsomal membrane fraction (Figure 1).

In anti-phosphotyrosine immunoprecipitates from the solubilized subcellular membrane fractions, PI 3-kinase activity in the PM-containing fraction was increased significantly by insulin (7.4-fold) but to a much greater extent by PDGF-bb (37.3-fold) (Table 2). However, in the microsomal membrane fraction in these same experiments this trend was reversed, with insulin being the predominant stimulator of PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates (Table 2) (4.6-fold stimulation), whereas PDGF-bb caused a smaller stimulation (2.3-fold). Surprisingly in these same three experiments PDGF-bb was found to cause a large increase (8.9-fold) in cytosolic PI 3-kinase activity whereas insulin had a much smaller effect (2.9-fold). The basis for this stimulation by PDGF-bb of cytosolic phosphotyrosine-associated PI 3-kinase activity is not clear and this activity may not be physiologically relevant in view of the lack of substrate in the cytosol.

PI 3-kinase activity was also assayed directly in membrane fractions (Figure 2). In these assays PI 4-kinase activity was also present, although the phosphatidylinositol 4-phosphate [PI(4)P] was resolved from phosphatidylinositol 3-phosphate [PI(3)P] on

Table 3 Effects of PDGF-bb and insulin in stimulating membrane-associated PI 3-kinase activity in subcellular membrane fractions of 3T3-L1 adipocytes

Overnight serum-starved cells were stimulated with PDGF-bb (20 ng/ml) or insulin (10 nM) for 10 min. Subcellular fractions were prepared and directly assayed for PI 3-kinase activity as described in the Materials and methods section, and the PI(3)P band was quantified using a BAS2000 Phosphorimager. Results are expressed as fmol of phosphate transferred per min per 10 cm dish of 3T3-L1 adipocytes. Results are the means ± S.E.M. for eight independent experiments.

	PI 3-kinase activity in membranes (fmol per min per dish)	
Stimulus	PM-containing fraction	Microsomal membranes
Basal	18.0 ± 2.0	50.6 ± 10.7
Insulin	40.0 ± 6.0	83.3 ± 11.3
PDGF-bb	44.0 ± 7.3	41.9 ± 6.7

the TLC system used. To determine which bands represented PI(3)P, parallel incubations were carried out in the presence of NP40 (results not shown) or wortmannin (Figure 2), both of which inhibit PI 3-kinase activity but not PI 4-kinase activity [9,22]. In some experiments a PI(4)P standard was also run. The results of a typical experiment investigating the effect of PDGF and insulin on PI 3-kinase activity in membrane fractions are shown in Figure 2.

The pattern of stimulation of PI 3-kinase activity in direct assays of membrane fractions was qualitatively similar to that seen in the anti-phosphotyosine immunoprecipitates, although the presence of a higher basal activity makes the fold stimulation lower, and differences in the assay system and lipid environment between the immunoprecipitate assay and the membrane assay mean that growth-factor-induced increases in PI 3-kinase activity cannot be directly compared quantitatively. However, in membrane assays the basal PI 3-kinase activity in the PM fraction was lower than in the LDM fraction. In eight separate experiments, PI 3-kinase activity in the PM fraction was stimulated by both PDGF (2.3-fold) and insulin (2.2-fold), whereas microsomal-membrane-associated PI 3-kinase activity was increased by insulin stimulation (1.7-fold) but not at all by PDGF-bb (Table 3). PI 4-kinase activity was predominantly located in the PM fraction and was not increased by either insulin or PDGF (Figure 2).

Presence of PI 3-kinase isoforms in 3T3-L1 membrane fractions

The presence of isoforms of the PI 3-kinase p85 subunit was investigated in the subcellular fractions. A polyclonal antibody raised against the N-terminal SH2 domain of p85α recognized a band of 85 kDa in PM-containing fractions, microsomes and cytosol (Figure 3). The 85 kDa band was also recognized by two other polyclonal antibodies raised against the N-terminal SH2 domain of p85α, by a polyclonal antibody raised against the SH3/bcr regions of p85α and by a monoclonal antibody (U13) that recognizes an epitope in the SH3 domain of human p85α (results not shown). Monoclonal antibodies raised against p85β and p85γ did not recognize any bands in 3T3-L1 adipocytes (results not shown). The majority (65–75%) of the p85α was found in the cytosol in both serum-starved and stimulated cells.

Interestingly, three separate polyclonal antisera raised against the glutathione S-transferase fusion proteins of the N-terminal SH2 domain of p85 all recognized a 65 kDa band in the PM-containing fraction (Figure 3). The 65 kDa band was not recognized by either of the antibodies raised against the SH3/bcr

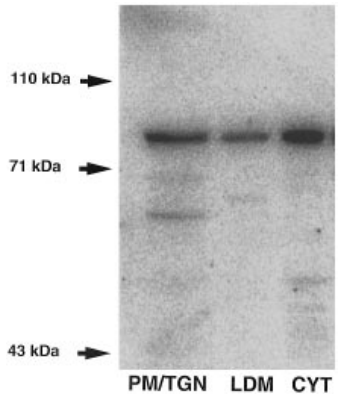

Figure 3 Identification of PI 3-kinase p85α subunit in subcellular fractions of 3T3-L1 adipocytes

Cytosol (CYT), PM-containing fraction (PM/TGN) and microsomal (LDM) fractions were prepared from serum-starved 3T3-L1 adipocytes. Equal amounts of each fraction were separated by SDS/PAGE and immunoblotted with a polyclonal antibody raised against the N-terminal SH2 domain of p85α followed by ^{125}I-labelled Protein A. The bands were revealed with a BAS2000 Phosphorimager.

Table 4 Growth-factor-stimulated recruitment of p85α to membrane fractions

Serum-starved 3T3-L1 adipocytes were incubated with insulin (10 nM) or PDGF-bb (20 ng/ml) for 10 min. Membrane fractions were prepared as described in the Materials and methods section, separated by SDS/PAGE and transferred to poly(vinylidene difluoride) filters. Filters were Western blotted with polyclonal antibody to the p85α subunit of PI 3-kinase. Results were quantified on a BAS2000 Phosphorimager and represent the average of five determinations in three separate experiments.

	p85α protein level (fold increase over basal)	
Stimulus	PM-containing fraction	Microsomal membranes
Insulin	1.11 ± 0.12	2.23 ± 0.35
PDGF-bb	1.47 ± 0.12	0.86 ± 0.15

domains (results not shown). This is of interest because a truncated homologue of p85 with similar immunological profile, referred to as p55^{PIK}, has recently been reported [23]. This protein contains regions highly homologous with the SH2 domains of p85 but lacks regions homologous with the SH3/BCR domains.

Growth-factor-stimulated recruitment of p85α to subcellular fractions

The effects of insulin and PDGF on the level of p85α in the PM-containing fraction and microsomal fractions qualitatively matched the effect of these growth factors on PI 3-kinase activity in the same membrane fractions. PDGF-bb increased p85α levels in the PM-containing fraction, but not in the microsomal fraction, whereas insulin caused a small increase in the level of p85α in the PM-containing fraction but a much larger increase (approx. 2-fold) in the microsomal fraction (Table 4).

Immunoblots of microsomal membrane fractions showed that recruitment of p85α to the microsomal fraction was maximal after approx. 10 min of stimulation with insulin (Figure 4). The insulin-stimulated recruitment of p85α to the microsomal fraction

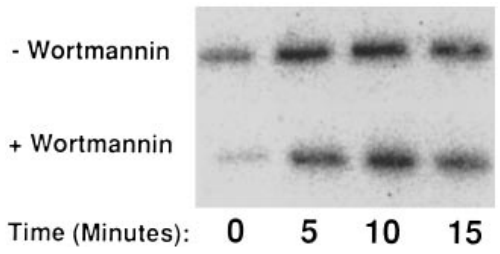

Figure 4 Effect of wortmannin on insulin-stimulated p85 recruitment to the microsomal membranes

Serum-starved 3T3-L1 adipocytes were incubated in 100 nM wortmannin or DMSO for 5 min before stimulation with 10 nM insulin for the time indicated. Microsomal fractions were prepared, separated by SDS/PAGE and immunoblotted with a polyclonal antibody raised against the N-terminal SH2 domain of p85α. Results show the 85 kDa band corresponding to p85α; similar results were obtained in three separate experiments.

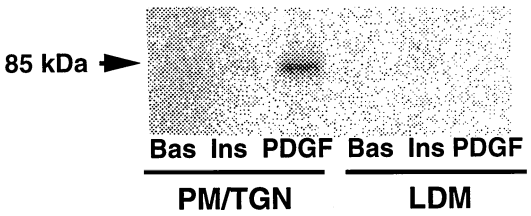

Figure 5 Tyrosine phosphorylation of p85 in response to growth factors

Serum-starved 3T3-L1 adipocytes were incubated with vehicle (lanes marked Bas), insulin (Ins; 10 nM) or PDGF-bb (20 ng/ml) for 10 min. PM-containing and microsomal fractions were prepared and immunoprecipated with an antibody raised to the N-terminal SH2 domain of p85α. Immunoprecipitates were run on SDS/PAGE and immunoblotted with PY20 anti-phosphotyrosine antibody. The bands were revealed with a BAS2000 phosphorimager.

was not affected by preincubation with 100 nM wortmannin (Figure 4), although this concentration of wortmannin completely blocked insulin-stimulated PI 3-kinase activity and insulin-stimulated glucose transport [24].

Tyrosine phosphorylation of p85 in response to growth factors

Phosphorylation of p85 was investigated by immunoprecipitation with p85 antibodies followed by anti-phosphotyrosine immunoblotting. As would be expected, tyrosine phosphorylation of p85 is absent in unstimulated 3T3-L1 adipocytes. PDGF stimulated tyrosine phosphorylation of p85 in the PM-containing fraction but had no effect on tyrosine phosphotylation of p85 in the microsomal fraction (Figure 5), whereas insulin did not stimulate tyrosine phosphorylation of p85 in either fraction.

DISCUSSION

Insulin stimulation of the translocation of both glucose transporter and transferrin receptor requires PI 3-kinase activity [4,8–11]. However, both PDGF and insulin stimulate PI 3-kinase activity in 3T3-L1 adipocytes, although PDGF barely stimulates glucose transport and does not stimulate transferrin receptor recruitment. It is therefore important to understand how growth factors are able specifically to regulate particular subsets of PI 3-kinase-dependent events in the cell. As the different PI 3-kinase-dependent responses occur at distinct sites within the cell, it is possible that specificity in signalling could be achieved by differential regulation of PI 3-kinase at specific cellular locations.

In the present study the effects of growth factors on PI 3-kinase activity in subcellular membrane fractions and anti-phosphotyrosine immunoprecipitates from these membrane fractions were investigated. These studies demonstrate that PDGF stimulation of PI 3-kinase is largely confined to the PM-containing fraction and cytosol rather than the microsomal fraction. In contrast, insulin stimulates PI 3-kinase to some extent in the PM-containing fraction but its major stimulatory effect is in the microsomal fraction. The effects of PDGF on intracellular PI 3-kinase activity have not previously been investigated in insulin-sensitive cell types, although there are several previous reports on the effects of insulin on subcellular PI 3-kinase activity in adipocytes. One of these studies reported no increase in microsomal PI 3-kinase activity after insulin stimulation [25], whereas two other studies [26,27] found insulin-stimulated PI 3-kinase activity in the microsomal fraction in adipocytes. However, the studies came to different conclusions as to the mechanism by which this increase in PI 3-kinase activity is achieved. In isolated rat adipocytes insulin did not cause an increase in microsomal p85 protein levels, although microsomal PI 3-kinase activity increased approx. 3-fold [26]. That finding was interpreted as an increase in PI 3-kinase 'relative catalytic activity', presumed to be due to the association observed between p85 and tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) [28]. In the present study the insulin- and PDGF-induced increases in membrane-associated PI 3-kinase activity in the PM-containing fraction are larger than the effect of these growth factors on recruitment of p85α. Therefore it could be argued that relative catalytic activity is being increased in the PM-containing fraction after insulin or PDGF stimulation. However, insulin increased microsomal PI 3-kinase activity and p85α levels to a similar extent, indicating that recruitment of PI 3-kinase to the microsomes is the major mechanism of insulin stimulation of PI 3-kinase activity in this fraction in these cells.

The pattern of localization of the p85 and PI 3-kinase activity after growth-factor stimulation could explain some of the similarities and differences in the actions of insulin and PDGF. For instance, the finding that both insulin and PDGF stimulate p85 recruitment and PI 3-kinase activity in the PM-containing fraction is consistent with the observation that both insulin and PDGF stimulate membrane ruffling, which is a PI 3-kinase-dependent event occurring at the PM [7,29]. Conversely, only insulin significantly stimulates p85 recruitment and PI 3-kinase activity in the microsomal fraction, which also contains the translocatable pool of glucose transporters. As glucose transporter translocation is a PI 3-kinase-dependent process [4,8–11,30] that is stimulated by insulin but not PDGF, this suggests that differences in stimulation of microsomal PI 3-kinase activity could provide an explanation for the differential effects of these two hormones on membrane trafficking events.

Little is known about the mechanism by which PI 3-kinase is recruited to the microsomal fraction, although it is presumed to be via tyrosine-phosphorylated IRS-1. As there is evidence to suggest that PI 3-kinase activity is necessary for the ligand-mediated sorting of internalized PDGF receptors [31] and also for protein traffic from the TGN to lysosomes [32], the possibility also exists that PI 3-kinase activity could play a role in its own intracellular targeting via regulating trafficking of a molecule such as IRS-1. Therefore an important finding of the present study is that wortmannin did not block the insulin-stimulated recruitment of p85 to the microsomal fraction, which rules out the involvement of PI 3-kinase in the mechanisms regulating recruitment of PI 3-kinase to intracellular membranes.

A further level of specificity in PI 3-kinase signalling is seen in the fact that PDGF but not insulin stimulates tyrosine phosphorylation of p85. This is in agreement with previous findings for insulin [23] and PDGF [33] in other cell types. The novel finding of the present study is that the tyrosine phosphorylation of p85 by PDGF is confined to the PM-containing fraction. This could have functional consequences for PDGF signalling at this site because it has previously been demonstrated that tyrosine phosphorylation of p85 affects the ability of its N-terminal SH2 domain to bind to the appropriate tyrosine-phosphorylated motif on the PDGF receptor [33].

In summary, the results of the present study demonstrate that in 3T3-L1 adipocytes PI 3-kinase activity is differentially regulated at the PM and in microsomal membranes in response to either PDGF or insulin. These differences might explain how both insulin and PDGF can stimulate PI 3-kinase and yet activate only specific subsets of PI 3-kinase-dependent responses.

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