Confocal imaging of the subcellular distribution of phosphatidylinositol 3,4,5-trisphosphate in insulin- and PDGF-stimulated 3T3-L1 adipocytes

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The activation of phosphatidylinositol 3-kinase (PI 3-kinase) and production of PtdIns(3,4,5) P_3 is crucial in the actions of numerous extracellular stimuli, including insulin-stimulated glucose uptake. Platelet-derived growth factor (PDGF) also stimulates PI 3-kinase, but only weakly promotes glucose uptake when compared with insulin. Insulin and PDGF have thus been proposed to have differential effects on the subcellular targeting of PI 3-kinase. However, owing to a lack of suitable methodologies, the subcellular localization of the PtdIns(3,4,5) P_3 generated has not been examined. The pleckstrin-homology (PH) domains of the nucleotide exchange factors, ADP-ribosylation factor nucleotide-binding-site opener (ARNO) and general receptor for 3-phosphoinositides (GRP1), which have a high affinity and specificity for PtdIns(3,4,5) P_3 , were fused to green fluorescent protein and used to examine the subcellular localization of PtdIns(3,4,5) P_3 generation in living 3T3-L1 adipocytes. PtdIns(3,4,5) P_3 was

produced almost exclusively in the plasma membrane in response to both agonists, although the response to insulin was greater in magnitude and occurred in considerably more cells. The results suggest that the greater ability of insulin to stimulate glucose uptake may be the result of its ability to generate significantly more plasma-membrane PtdIns(3,4,5) P_3 than PDGF. ARNO and GRP1 are nucleotide exchange factors for the small GTP-binding protein ADP-ribosylation factor 6 (ARF6). The inability of a constitutively active GTPase-deficient mutant of ARF6 (ARF6-Q67L; Gln⁶⁷ → Leu) to cause glucose transporter GLUT4 translocation suggests that activation of this pathway is not sufficient to cause GLUT4 translocation.

Key words: ADP-ribosylation factor, glucose transport, growth factor, insulin, phosphatidylinositol 3-kinase.

INTRODUCTION

The class I phosphatidylinositol 3-kinases (PI 3-kinases) comprise a regulatory subunit (M_r 85000) and a catalytic subunit (M_r 110000) which phosphorylates PtdIns(4,5) P_2 on the 3-position of the inositol ring to produce PtdIns(3,4,5) P_3 . This class of enzyme is activated by a wide variety of extracellular stimuli [1,2] and plays a crucial role in the regulation of multiple cellular processes such as cell survival [3], cytoskeletal rearrangements [4] and insulin-stimulated glucose uptake [5,6].

Growth factors, such as platelet-derived growth factor (PDGF), stimulate PI 3-kinase by promoting its association with Y(P)XXM motifs in the intracellular domain of their receptors – the tyrosine being autophosphorylated after ligand binding [1]. In contrast, insulin activates PI 3-kinase by promoting its interaction with Y(P)XXM motifs that are phosphorylated on the insulin-receptor substrates (IRSs) IRS-1 and IRS-2 by the insulin-receptor tyrosine kinase (see [7] for review).

It is becoming increasingly clear that the subsequent interaction of PtdIns(3,4,5) P_3 with proteins occurs at least in part through an approx. 100-amino-acid protein module termed a pleckstrin homology (PH) domain [1,8]. For example, protein kinase B (PKB/Akt) contains a PH domain which interacts with PtdIns(3,4,5) P_3 , allowing it to translocate to the plasma membrane and become activated by phosphorylation on threonine-

308 and serine-473 by 3-phosphoinositide-dependent kinase-1 (PDK1) and PDK2 respectively [9,10]. PDK1 itself has a PH domain and also plays an important role in the activation of p70 S6 kinase [11,12]. PDK2 has yet to be formally identified.

Some studies have reported a stimulus-dependent localization of active PI 3-kinase to distinct subcellular compartments, suggesting that protein function or vesicle trafficking may be differentially regulated depending on the site of generation of PtdIns(3,4,5) P_3 . For example, in adipocytes, insulin and PDGF both stimulate total cellular PI 3-kinase activity to a similar extent. Insulin preferentially increases PI 3-kinase activity in intracellular vesicles that contain the GLUT4 isoform of the glucose transporter family [13–15], leading to the proposal that the specificity behind the ability of insulin, but not PDGF, to stimulate glucose uptake via a translocation of GLUT4 to the plasma membrane (reviewed in [16]), lies in the subcellular targeting of PI 3-kinase to GLUT4 vesicles [13–15]. IRS-1 family members may play an essential role in such targeting, as they are not substrates for phosphorylation by the PDGF receptor and are predominantly found associated with membranes in adipocytes [17,18].

Current methods of PtdIns(3,4,5) P_3 detection only allow measurements of the total cellular synthesis and mass of PtdIns(3,4,5) P_3 , and cannot easily address its subcellular localization. To circumvent this problem, and to provide a dynamic

Abbreviations used: GFP, green fluorescent protein; PI 3-kinase, phosphatidylinositol 3-kinase; ARNO, ADP-ribosylation factor (ARF) nucleotide binding site opener; GRP1, general receptor for 3-phosphoinositides; IRS, insulin-receptor substrate; PDGF, platelet-derived growth factor; PDK, 3-phosphoinositide-dependent kinase; PH, pleckstrin homology; IRAP, insulin-responsive aminopeptidase; PKB/(Akt), protein kinase B; TRITC, tetramethylrhodamine isothiocyanate; GroP, glycerophospho; HA, haemagglutinin; DMEM, Dulbecco's modified Eagle's medium.

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assay for $\text{PtdIns}(3,4,5)P_3$ production, we sought to develop a single-cell assay for $\text{PtdIns}(3,4,5)P_3$. This is based on the use of the PH domains of two distinct guanine nucleotide exchange factors for the ADP-ribosylation factor (ARF) family called ARF-nucleotide binding site opener (ARNO; [19]) and general receptor for 3-phosphoinositides (GRP1; [20]) [21].

We recently reported that the PH domain of recombinant ARNO binds $\text{Ins}(1,3,4,5)P_4$ and glycerophospho (GroP)- $\text{PtdIns}(3,4,5)P_3$ (in which the 1-phosphate is esterified with glycerol), through its PH domain with K_d values of approx. 70 and 88 nM respectively [22]. Furthermore, the PH domain of ARNO binds $\text{Ins}(1,3,4,5,6)P_5$, $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ with at least 100-fold lower affinity ($K_d > 1000$ nM). In a similar fashion, recombinant glutathione S-transferase–GRP1 bound $\text{Ins}(1,3,4,5)P_4$ and $\text{GroPIns}(3,4,5)P_3$ with K_d values of 32 nM and 38 nM respectively; $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4)P_3$ bound with K_d values > 10000 nM [23]. This binding activity was dependent on the PH domain of GRP1 and is very similar to the *in vitro* binding data reported for $\text{PtdIns}(3,4,5)P_3$, $\text{PtdIns}(3,4)P_2$ and $\text{PtdIns}3P$ [20].

We have reported that a full-length ARNO and GRP1, tagged at the N-terminus with green fluorescent protein (GFP), exhibit a rapid translocation to the plasma membrane in response to insulin stimulation of 3T3-L1 adipocytes and nerve-growth-factor stimulation of PC12 cells respectively [22,23]. These effects were completely reversed by the PI 3-kinase inhibitors wortmannin, LY294002 and a dominant-negative PI 3-kinase ($\Delta p85$) [22,23]. On the basis of this we proposed that the PH domains of ARNO and GRP1 constitute *bona fide* $\text{PtdIns}(3,4,5)P_3$ receptors.

In the present study we have utilized the PH domains of ARNO and GRP1, as fusion proteins with GFP, to detect the synthesis of $\text{PtdIns}(3,4,5)P_3$ in single living 3T3-L1 adipocytes in response to insulin and PDGF. We also investigated a potential role for ARF6 in GLUT4 translocation, as this small GTP-binding protein is a proposed *in vivo* substrate for ARNO and GRP1.

MATERIALS AND METHODS

Materials

A murine 3T3-L1 fibroblast clone was obtained from the American Tissue Culture Collection (catalogue no. CCL 92.1). Tissue-culture reagents were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.) or Sigma Chemical Company (Poole, Dorset, U.K.). Porcine PDGF was from Calbiochem (Nottingham, U.K.).

Plasmids

GFP fused either to full-length human ARNO, or the ARNO PH domain (amino acids 262–399) were as described in [22]. Similarly, GFP fused to either full-length human GRP1 or the isolated PH domain (amino acids 267–399) were as described in [23]. The ARF6-Q67L and ARF6-T27N mutants in the plasmid pXS [24] were generously supplied by Dr. Julie Donaldson (NIH, Bethesda, MD, U.S.A.). GFP fused to full-length GLUT4 was as described in [25].

Cell culture, adipocyte differentiation and microinjection

3T3-L1 adipocytes cultured on polylysine-coated glass coverslips were microinjected with plasmids at 100 $\mu\text{g}/\text{ml}$ as described in [25]. After microinjection the cells were incubated at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) Myoclon-Plus foetal-calf serum for 16–24 h. Before treat-

ments and fluorescence image analysis, microinjected adipocytes were incubated for 2 h at 37 °C in serum-free DMEM, and then for the times indicated in the Figure legends in the presence of 200 nM insulin or 100 ng/ml PDGF, as required.

Immunofluorescence and confocal microscopy

In some experiments the cellular distribution of the insulin-responsive aminopeptidase (IRAP) was determined. Cells, fixed and permeabilized using 4% paraformaldehyde and 1% Triton X-100, were stained with rabbit polyclonal anti-IRAP antibodies (5 $\mu\text{g}/\text{ml}$; kindly provided by Dr. Susanna Keller and Dr. Gus Lienhard, Dartmouth Medical School, Hanover, NH, U.S.A.) in PBS containing 3% (w/v) BSA. This was followed by incubation in a 1:100 dilution of tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-(rabbit IgG) for 15 min (Vector Laboratories, Peterborough, U.K.). In other experiments, fixed and permeabilized cells were immunostained with monoclonal anti-haemagglutinin (anti-HA) antibodies (10 $\mu\text{g}/\text{ml}$ of HA11; Berkeley Antibody Company, Richmond, CA, U.S.A.) for 30 min, followed by incubation in a 1:200 dilution of FITC-conjugated goat anti-(mouse IgG) (Sigma) for 30 min. Confocal microscopy and image analysis was performed as described in [22].

Co-precipitation of PI 3-kinase in anti-phosphotyrosine immunoprecipitates

Cells were serum-starved for 2 h and treated with insulin (200 nM) or PDGF (100 ng/ml) for 2 min and extracted in 0.5 ml of 50 mM Hepes/0.2 mM EDTA/2.2 mM EGTA/100 mM KCl/10% glycerol/1% Triton X-100. Clarified extracts were immunoprecipitated with anti-phosphotyrosine antibody (1:100 dilution; clone PY20, ICN) coupled to Protein A–Sepharose. Proteins were separated on an SDS/8%-polyacrylamide gel and then transferred to Immobilon P membrane (Millipore) using a semi-dry transblotter (Bio-Rad). Western blotting was performed using a 1:1000 dilution of rabbit polyclonal anti-p85 antibody (a gift from Dr. Peter Shepherd, Department of Biochemistry and Molecular Biology, University College London, London, U.K.) followed by detection with a 1:10000 dilution of donkey anti-(rabbit IgG) conjugated to horseradish peroxidase (Amersham) followed by enhanced chemiluminescence (ECL[®]) detection (Amersham).

Western analysis of PKB phosphorylation at Ser⁴⁷³

Confluent 3T3-L1 adipocytes in 35 mm-diameter dishes were treated under the conditions described in the Figure legends, lysed and subjected to Western blotting with rabbit polyclonal Phospho-specific Akt (Ser⁴⁷³) antiserum (New England Biolabs, Beverly, MA, U.S.A.) as described in [26].

RESULTS

Development of a single-cell $\text{PtdIns}(3,4,5)P_3$ assay

As GLUT4 translocation occurs with a half-life of approx. 2.4 min in 3T3-L1 adipocytes [16], we examined these cells during a 10 min stimulation period with insulin. Cells were microinjected with a plasmid possessing a cDNA encoding the PH domain of ARNO (GFP–ARNO^{PH}) under the control of the strong cytomegalovirus promoter. After 24 h the cells were serum-starved, then treated with insulin, and finally fixed, permeabilized and stained with an antibody to the GLUT4 vesicle resident protein IRAP. IRAP co-localizes with GLUT4 and has been reported to exhibit a pronounced insulin-

Figure 1 Insulin stimulates the translocation of GFP-ARNO to the plasma membrane of 3T3-L1 adipocytes

3T3-L1 adipocytes were microinjected with a plasmid encoding GFP tagged to the N-terminus of either the PH domain of ARNO (GFP-ARNO^{PH}; **A**) or full-length ARNO (GFP-ARNO; **B**). After 24 h the cells were serum-starved and then incubated with 200 nM insulin for 0, 2, 5 or 10 min as indicated. The cells were then fixed with 4% paraformaldehyde and permeabilized with 1% Triton for staining with anti-IRAP antibodies (**A** only) and observation by confocal microscopy. In (**A**) the distribution of GFP-ARNO^{PH} (left column) and IRAP (right column) is shown. A considerable amount of GFP-ARNO^{PH} appears to localize in the nucleus, probably due to its free access via the nuclear pore. As a result there is a small amount of bleed-through of the GFP in the TRITC channel. In (**B**) the distribution of GFP-ARNO is shown.

Figure 2 Insulin stimulates the translocation of GFP–GRP1 to the plasma membrane of 3T3-L1 adipocytes

3T3-L1 adipocytes were microinjected with a plasmid encoding GFP fused to the N-terminus of either the PH domain of GRP1 (GFP–GRP1^{PH}) or full-length GRP1 (GFP–GRP1). After 24 h the cells were serum-starved and then incubated with 200 nM insulin for 0, 2, 5 or 10 min, as indicated, and then fixed with 4% paraformaldehyde. The distribution of GFP–GRP1^{PH} (left column) and GFP–GRP1 (right column) was examined by confocal microscopy.

dependent translocation to the plasma membrane along with GLUT4 [27–30].

As demonstrated in Figure 1(A), in the basal state the majority of GFP–ARNO^{PH} was found in the cytosol of these cells and was excluded from the fat droplets. The protein also appeared to enter the nucleus, a phenomenon which is also observed with native GFP and is almost certainly the result of its ability to freely enter through the nuclear pore as a result of its size (approx. 37 kDa). Consistent with this, full-length ARNO tagged

at the N-terminus with GFP was excluded from the nucleus (Figure 1B).

Insulin caused an almost complete translocation of GFP–ARNO^{PH} to the plasma membrane, an effect which was sustained for at least 10 min (Figure 1A). Careful examination of cells expressing GFP–ARNO^{PH} revealed no increase in fluorescence of any intracellular vesicle, including those immunoreactive with the anti-IRAP antibody (Figure 1A). This strongly suggests that the bulk of insulin-stimulated PtdIns(3,4,5) P_3 generation occurs at the plasma membrane and not to a detectable extent in the GLUT4 vesicles themselves.

As the GFP–ARNO^{PH} chimera is significantly expressed in the nucleus, we repeated these experiments using the full-length GFP–ARNO fusion protein which exhibits a considerably brighter cytoplasmic fluorescence, thus increasing the sensitivity of the technique. As shown in Figure 1(B), the result obtained with GFP–ARNO was almost indistinguishable from that we observed with the PH domain alone; again we saw no evidence for the appearance of fluorescence in intracellular vesicles in response to insulin. Thus, importantly, the ARNO Sec7 domain does not appear to influence the subcellular targeting of the PH domain, thus making it a suitable probe for the presence of PtdIns(3,4,5) P_3 in the cell (note that a GFP fusion with the Sec7 domain of ARNO is cytosolic and does not exhibit any alteration in subcellular localization with insulin [22]).

In order to confirm these results, full-length GRP1 tagged at the N-terminus with GFP (GFP–GRP1) was expressed in 3T3-L1 adipocytes by microinjection. In the basal state GFP–GRP1 was exclusively expressed in the cytoplasm. Insulin caused a pronounced and almost complete translocation of GFP–GRP1 to the plasma membrane in these cells, which was again sustained for the entire 10 min period of stimulation we examined (Figure 2). Almost indistinguishable results were obtained with GFP tagged to the N-terminus of the isolated PH domain of GRP1 (Figure 2). This translocation was dependent on the presence of the PH domain, as a fusion protein between the GRP1 Sec7 domain and GFP was expressed in the cytoplasm and exhibited no change in distribution upon insulin stimulation (results not shown).

The insulin-dependent translocation of GFP–GRP1 occurred in 93 % of the cells ($n = 74$ cells) and was substantially blocked when we prevented the activation of PI 3-kinase by a pre-incubation for 15 min in the presence of 100 nM wortmannin (translocation occurred in 16 % of the cells, $n = 19$), 50 μ M LY294002 (14 %, $n = 21$) or by co-injection of 100 μ g/ml of a plasmid encoding a dominant-negative PI 3-kinase ($\Delta p85$; 19 %, $n = 42$). Taken together, this strongly suggests that GRP1, like ARNO, is a *bona fide in vivo* PtdIns(3,4,5) P_3 receptor. Consistent with this, the effect of insulin on GFP–ARNO translocation was maximal at 10 nM insulin, with a half-maximal effect at approx. 3–5 nM (results not shown). This is similar to the half-maximal effect of insulin on PI 3-kinase activation as reported by Lamphere and co-workers [31].

As with the GFP-tagged ARNO constructs, we observed no increase in fluorescence of any intracellular vesicles in response to insulin when using GFP-tagged GRP1 constructs, strongly suggesting that the bulk of the PtdIns(3,4,5) P_3 generated in response to insulin occurs in the plasma membrane. Thus we can find no *in vivo* evidence for the presence PtdIns(3,4,5) P_3 in GLUT4 vesicles using four GFP-tagged probes (GFP–ARNO, GFP–ARNO^{PH}, GFP–GRP1 or GFP–GRP1^{PH}), although we cannot exclude the possibility that insulin induces a small rise in PtdIns(3,4,5) P_3 levels in GLUT4 vesicles that is below the level of sensitivity of our assay. However, taken together, these constructs provide a dynamic *in vivo* tool for the detection of PtdIns(3,4,5) P_3 .

Figure 3 Differential effects of insulin and PDGF on PtdIns(3,4,5) P_3 production in the plasma membrane of 3T3-L1 adipocytes

3T3-L1 adipocytes were microinjected with a plasmid encoding GFP-ARNO. After 24 h the cells were serum-starved, incubated at 37 °C on the stage of the confocal microscope in Hepes-buffered Krebs medium (as described in [24]), and images were collected at 20 s intervals. PDGF (100 ng/ml) was added immediately prior to the collection of image (c), and then insulin (200 nM) added immediately prior to the collection of image (l). Images (a)–(k) are sequential images. Images (l)–(o) were also collected sequentially, but 160 s after the collection of image (k). Image (p) identifies the cells (A–E) discussed in the text. The full sequence should be viewed as a time-lapse animation which can be accessed using either Netscape (version 3 or higher) or Internet Explorer (version 4 or higher) at the following URL: <http://www.BiochemJ.org/bj/344/bj3440511add.htm>

at the single-cell level which is currently very difficult to achieve using available biochemical techniques.

Insulin increases plasma-membrane PtdIns(3,4,5) P_3 to a greater extent than PDGF

As GFP-tagged full-length ARNO was excluded from the nucleus

and exhibits a behaviour which was otherwise indistinguishable from the ARNO and GRP1 PH domains, we used this as a tool to detect the subcellular localization of PtdIns(3,4,5) P_3 generated in response to insulin and PDGF in 3T3-L1 adipocytes using time-lapse confocal microscopy (Figure 3). This experiment is best viewed in conjunction with an animated version of Figure 3 which can be accessed via the URL: <http://>

Figure 4 Insulin stimulates PI 3-kinase-dependent phosphorylation of PKB on Ser⁴⁷³ to a greater extent than PDGF

(A) 3T3-L1 adipocytes were serum-starved and incubated in the absence (C) or presence of 200 nM insulin (I) or 100 ng/ml PDGF (P) for 10 min. The cells were lysed and the extent of co-precipitation of the p85 subunit of PI 3-kinase with phosphotyrosine-containing proteins was determined by Western blotting anti-phosphotyrosine immunoprecipitates with anti-p85 antibodies. (B) Serum-starved 3T3-L1 adipocytes were pre-incubated with 100 nM wortmannin for 10 min, or 50 μ M LY294002 for 15 min, as indicated. The cells were then incubated in the absence (C) or presence of 200 nM insulin (I) or 100 ng/ml PDGF (P) for a further 10 min. The level of PKB phosphorylation on Ser⁴⁷³ was measured by Western blotting cell lysates with a rabbit Phospho-specific Akt (Ser⁴⁷³) antiserum ('Anti-Ser473'). The blot was stripped and re-probed with a rabbit anti-PKB antiserum ('Anti-PKB').

www.BiochemJ.org/bj/344/bj3440511add.htm [use Netscape (version 3 or higher) or Internet Explorer (version 4 or higher)].

The addition of PDGF caused a small translocation of GFP-ARNO to the plasma membrane of some cells (e.g., see cell 'C' in images c-k of Figure 3). However, PDGF rarely caused the formation of a complete ring of GFP-ARNO fluorescence around the plasma membrane, as was observed when insulin was subsequently added (e.g. cells 'A', 'D' and 'E' of panels l-o in Figure 3). Insulin, and to some extent PDGF, caused the appearance of GFP-ARNO in membrane ruffles (e.g., cell 'D' of panels l-o of Figure 3). Interestingly, this apparent localization of PtdIns(3,4,5)P₃ to membrane ruffles is consistent with the reported involvement of PI 3-kinase in this process, at least in fibroblasts in response to PDGF [4]. The response to PDGF took at least 40 s to initiate and was not maximal until 80 s. In contrast, insulin-dependent translocation initiated within 20 s, was complete within 40 s (compare the response of cell 'C' with that of cells 'A', 'D' and 'E' in Figure 3; but see also [22]) and was greater in magnitude than the PDGF effect. In this experiment we again found no evidence for the appearance of GFP-ARNO on intracellular vesicles in response to insulin (even during a further 15 min incubation with insulin; results not shown).

In a separate experiment we confirmed that the recruitment of the p85 subunit of PI 3-kinase into anti-phosphotyrosine immunoprecipitates was induced to equivalent extents by insulin and PDGF (Figure 4A) as previously reported (see the Introduction).

Insulin stimulates PKB phosphorylation on Ser⁴⁷³ to a greater extent than PDGF

As insulin stimulates the generation of significantly more plasma membrane PtdIns(3,4,5)P₃ than PDGF, we would predict that insulin should stimulate the activation of PtdIns(3,4,5)P₃-dependent events to a greater extent than PDGF. A known PtdIns(3,4,5)P₃-dependent event is the stimulation of PKB

Figure 5 ARF6-Q67L does not cause GLUT4 translocation

3T3-L1 adipocytes were microinjected with plasmids encoding GFP-GLUT4 (A and B) or GFP-GLUT4 with ARF6-Q67L (C and D) or ARF6-T27N (E and F). After 24 h the cells were serum-starved for 2 h and then treated for 1 h in the absence (A, C-E) or presence (B) of 200 nM insulin for 1 h. The cells were fixed and stained with monoclonal anti-HA antibodies (C-F) followed by detection using FITC-conjugated goat anti-mouse IgG. The Figure shows confocal micrographs of representative cells. The GFP-GLUT4 distribution is shown in (A)-(C) and (E). The distribution of HA-tagged ARF6 is shown in (D) and (F).

activity, which occurs via phosphorylation of Thr³⁰⁸ and Ser⁴⁷³ by PDK1 and 'PDK2' respectively [11]. As the identity of 'PDK2' has not been unequivocally demonstrated [32,33], we measured phosphorylation of Ser⁴⁷³ by taking advantage of an anti-phosphopeptide antiserum that recognizes PKB only when phosphorylated at this residue. As shown in Figure 4(B), the basal level of phosphorylation of Ser⁴⁷³ was undetectable in 3T3-L1 adipocytes and that this was substantially increased by insulin and prevented by pre-incubation with the PI 3-kinase inhibitors wortmannin and LY294002. As predicted, PDGF caused a considerably lower increase in Ser⁴⁷³ phosphorylation than insulin which was also blocked by wortmannin and LY294002 (Figure 4B).

Lack of effect of ARF6 mutants on GLUT4 translocation

As ARNO and GRP1 are the likely *in vivo* exchange factors for ARF6 [34,35] we investigated the effect on GLUT4 distribution

in 3T3-L1 adipocytes of over-expressing either ARF6-Q61L (GTPase-defective, thus constitutively active and GTP-bound) or ARF6-T27N (GTP-binding defective, thus constitutively GDP-bound). The subcellular distribution of these proteins was determined by staining with an anti-HA epitope tag antibody. As shown in Figure 5, HA-tagged ARF6-Q67L was predominantly localized to the plasma membrane and ARF6-T27N was intracellular (compare Figures 5d and 5f). This is consistent with the known distribution of these ARF6 mutants and the fact that GTP loading of ARF6 causes it to translocate to the plasma membrane [24].

The distribution of GLUT4 in these cells was examined by co-expression with a GFP-tagged GLUT4 [25]. Insulin causes a pronounced translocation of GFP-GLUT4 to the plasma membrane of 3T3-L1 adipocytes (Figures 5a and 5b). However, neither ARF6-Q67L nor ARF6-T27N overexpression had any apparent effect on the distribution of GFP-GLUT4 (compare Figures 5c and 5e versus 5a).

DISCUSSION

In the present study we have demonstrated a rapid and profound insulin-stimulated translocation of ARNO and GRP1 to the plasma membrane of 3T3-L1 adipocytes. This translocation occurred regardless of whether we used the full-length protein or just the isolated PH domain (Figures 1 and 2) and did not occur if the PH domain was deleted (results not shown). The PH domains of these two proteins exhibit a high degree of specificity for the 3,4,5 configuration around the inositol ring and are, therefore, useful *in vivo* sensors of the presence of PtdIns(3,4,5) P_3 [22,23]. When used in combination with GFP and confocal microscopy, this provides a dynamic assay for the production of PtdIns(3,4,5) P_3 at the subcellular level, which is not possible using available biochemical techniques.

We examined the stimulation of PtdIns(3,4,5) P_3 levels in 3T3-L1 adipocytes by insulin and PDGF, as the former stimulus has been reported to preferentially increase PI 3-kinase activity in intracellular vesicles that contain the GLUT4 isoform of glucose transporter [13,14]. This model is thought to underlie the ability of insulin, but not PDGF, to stimulate glucose uptake.

The insulin-dependent translocation of GFP-ARNO and GFP-GRP1 to the plasma membrane (and their isolated PH domains) was rapid and sustained for at least 10 min. We also expected to see an increase in PtdIns(3,4,5) P_3 production in GLUT4 vesicles. However, this was clearly not the case, as we saw no translocation of any of the ARNO or GRP1 constructs to intracellular vesicles that were immunoreactive with antibodies that recognize the GLUT4 vesicle resident protein IRAP (Figures 1–3). Thus the data suggest that the large bulk of insulin-dependent PtdIns(3,4,5) P_3 generation is restricted to the plasma membrane.

Using GFP-ARNO as a probe, both insulin and PDGF-stimulated a rise in plasma-membrane PtdIns(3,4,5) P_3 (Figure 3); however, the characteristics of this rise appeared to differ in three main respects: (i) the rise was slower to initiate in response to PDGF (40 s as against 20 s for insulin); (ii) only a small fraction of cells responded to PDGF (< 10 % as against > 90 % for insulin); and (iii) insulin stimulation appeared to result in the generation of more plasma-membrane PtdIns(3,4,5) P_3 than was produced in response to PDGF. This is in general agreement with biochemical measurements of total cellular PtdIns(3,4,5) P_3 production by labelling cells with [32 P] P_i [36].

PKB, which is activated by insulin in a PI 3-kinase-dependent manner, has been proposed to play an important role in insulin-dependent GLUT4 translocation [37,38]. We predicted that the

phosphorylation of this enzyme on Ser⁴⁷³, which is also a PI 3-kinase-dependent event, would occur to a greater extent in response to insulin than PDGF, and indeed this was the case (Figure 4B).

Taken together, our data suggest that insulin may cause GLUT4 translocation and glucose uptake, not because it targets PI 3-kinase to GLUT4 vesicles, but because it promotes a significantly greater increase in plasma-membrane PtdIns(3,4,5) P_3 levels than PDGF. Our results are in general agreement with the recent study by Frevert et al. [39], who reported that a constitutively active PI 3-kinase targeted to the GLUT4 vesicle did not promote glucose uptake or GLUT4 translocation. However, we cannot exclude the possibility that insulin, but not PDGF, results in the generation of other PI 3-kinase products or PtdIns(3,4,5) P_3 metabolites in GLUT4 vesicles, such as PtdIns(3,4) P_2 . This inositol lipid does not bind efficiently to the ARNO or GRP1 PH domains, and thus would not be detectable in this study. This possibility will require further testing when PtdIns(3,4) P_2 -specific probes become available.

How can we reconcile the fact that PDGF and insulin both stimulate PI 3-kinase in the plasma membrane, but PDGF stimulation results in the generation of significantly less PtdIns(3,4,5) P_3 ? It is possible that insulin stimulates the activation of a PI 3-kinase isoform that exhibits a considerably higher specific activity towards PtdIns(4,5) P_2 than an isoform stimulated by PDGF. Resolution of this issue will require the generation of PI 3-kinase catalytic-subunit isoform-specific antisera. More likely, perhaps, insulin may stimulate PI 3-kinase in a manner that allows it greater access to its substrate in the plasma membrane. Recent studies suggest that the insulin-stimulated PI 3-kinase may reside not on the GLUT4 vesicle itself, but in a closely associated cytoskeletal fraction [18], perhaps containing actin. The only cytoskeleton detectable in 3T3-L1 adipocytes is a cortical actin network immediately underneath the plasma membrane; otherwise the actin cytoskeleton is highly fragmented [25,40]. Thus it is possible that insulin stimulates PI 3-kinase in the underlying cytoskeleton (perhaps via its interaction with IRS-1), allowing it improved access to its substrate, PtdIns(4,5) P_2 . Alternatively, PDGF may be more potent than insulin at stimulating a counteracting PtdIns(3,4,5) P_3 phosphatase such as the 5'-phosphatidylinositide phosphatase SHIP [41]. Thus any PtdIns(3,4,5) P_3 generated in response to PDGF would be immediately dephosphorylated to PtdIns(3,4) P_2 , which would bind very poorly to the PH domains of ARNO or GRP1 [22,23]. Again this possibility will require further investigation using a PtdIns(3,4) P_2 -specific probe, and/or metabolic labelling of inositol phospholipids *in vivo*.

Whatever the explanation, using this single-cell assay for PtdIns(3,4,5) P_3 production we propose that the ability of insulin to stimulate GLUT4 translocation, and thus glucose transport, may be the result of its ability to generate significantly more plasma-membrane PtdIns(3,4,5) P_3 . This single-cell assay could be applied to a number of other important biological questions surrounding the role of PI 3-kinase in cell signalling, such as its role in cell-cell adhesion, endocytosis and growth control.

ARNO [34] and GRP1 [35] are nucleotide exchange factors for ARF6. Interestingly, Millar and co-workers have recently reported that inhibition of ARF6 function, using a myristoylated peptide corresponding to the N-terminus of ARF6, blocks insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes [42]. The fact that insulin stimulates the translocation of GRP1 and ARNO to the plasma membrane to a greater extent than PDGF suggests that the recruitment of these exchange factors to the plasma membrane might allow the activation of ARF6 (via GTP loading), thereby recruiting GLUT4 vesicles to the cell

surface. However, we have found that a constitutively active GTP-bound mutant of ARF6 (ARF6-Q67L) had no apparent effect on the translocation of GFP-tagged GLUT4 (Figure 5) despite the fact that this mutation induced plasma-membrane localization of ARF6, as expected (Figure 5d)[24]. Taken together the data suggest that ARF6 activation may be necessary, but not sufficient, to cause GLUT4 translocation in response to insulin. However, while this paper was under review, Mueckler and colleagues reported that a dominant-negative ARF6 blocked insulin-stimulated adipon secretion but not glucose uptake [43], which further suggests that ARF6 does not have a direct role in insulin-stimulated GLUT4 translocation. This conclusion is also consistent with the lack of effect of inhibitory GRP1 mutants or anti-GRP1 antibodies on insulin-stimulated glucose uptake [35]. Further work is necessary, therefore, to delineate the precise role of the ARF6 in the regulation of vesicle trafficking by insulin.

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