The pleckstrin homology domains of protein kinase B and GRP1 (general receptor for phosphoinositides-1) are sensitive and selective probes for the cellular detection of phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate *in vivo*

Alexander GRAY, Jeroen VAN DER KAAAY and C. Peter DOWNES

Department of Biochemistry, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

We have tested the binding specificities of the pleckstrin homology (PH) domains of protein kinase B (PKB) and GRP1 (general receptor for phosphoinositides-1), expressed as green fluorescent protein (GFP) fusion proteins [PH(PKB)GFP and PH(GRP1)GFP respectively] in HEK 293 cells and Swiss 3T3 cells, using confocal microscopy. Stimulation of HEK 293 cells with insulin caused a small, but sustained, increase in PtdIns(3,4,5) P_3 levels, detected using a radioligand displacement assay, which was mirrored by the translocation of PH(PKB)GFP and PH(GRP1)GFP from the cytosol to the plasma membrane of live, transfected cells. Similar results were obtained using Swiss 3T3 cells stimulated with platelet-derived growth factor (PDGF) and expressing either PH(PKB)GFP or PH(GRP1)-

GFP. Biochemical analyses confirmed the accumulation of both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 in response to PDGF, but only the latter was present at increased levels in Swiss 3T3 cells 30 min after an oxidative stress (1 mM H_2O_2). Concomitantly, only PH(PKB)GFP, and not PH(GRP1)GFP, was localized at plasma membranes after 30 min of treatment with H_2O_2 . The fusion proteins appear accurately to report the spatial and temporal distribution of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 in intact cells. These results establish the lipid selectivity of these PH domains *in vivo*, and further emphasize the overlapping, but distinct, second messenger roles of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 .

Key words: green fluorescent protein, confocal microscopy.

INTRODUCTION

Activation of a wide variety of cell surface receptors for hormones and growth factors results in the subsequent activation of type I phosphoinositide 3-kinases (PI 3-kinases), which phosphorylate PtdIns(4,5) P_2 to produce the lipid second messenger PtdIns(3,4,5) P_3 (reviewed in [1,2]). Accumulation of PtdIns(3,4,5) P_3 is usually accompanied by the production of PtdIns(3,4) P_2 , due either to the action of 5-phosphatases, which hydrolyse the former lipid, or via the action of type II PI 3-kinases. The latter may act directly on PtdIns4P or indirectly, via the synthesis of PtdIns3P and subsequent phosphorylation by PI 4-kinases [3,4]. Many cellular responses downstream of receptor activation have been shown to be dependent on the production of PtdIns(3,4,5) P_3 (see [5] for an extensive review).

In vitro binding studies have shown that the pleckstrin homology (PH) domains of some proteins, such as GRP1 (general receptor for phosphoinositides-1), bind PtdIns(3,4,5) P_3 with high affinity and selectivity [6]. Another group of PH domains, exemplified by those occurring in protein kinase B (PKB) and phosphoinositide-dependent kinase 1 (PDK1), appear to bind both to PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 [7–9]. One of the best studied examples is PKB, which possesses a single PH domain at its N-terminus. PKB is activated, in a PI 3-kinase-dependent manner, by phosphorylation at Thr-308 and Ser-473 catalysed by PDK1 [10]. PtdIns(3,4,5) P_3 appears to act in at least three ways in this system. First, PtdIns(3,4,5) P_3 binding appears to lead to translocation of PKB from the cytosol to the plasma membrane of stimulated cells [8,9]. Secondly, PtdIns(3,4,5) P_3

binding is required for PKB to become an effective substrate for PDK1. Thirdly, and most controversially, binding of PtdIns(3,4,5) P_3 to the C-terminally located PH domain of PDK1 stimulates the latter's catalytic activity when PDK1 is bound to a putative regulatory polypeptide. Binding of the latter polypeptide also converts PDK1 from a kinase that phosphorylates only Thr-308 of PKB into one that also phosphorylates Ser-473 [11].

The PH domains of PKB and PDK1 have been shown to bind both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 *in vitro*, but they have much lower affinities for PtdIns(4,5) P_2 [12]. Other well characterized PH domains, such as that found in phospholipase $C\delta$ -1, bind PtdIns(4,5) P_2 with higher affinity than the 3-phosphoinositides [13]. A third group of proteins possesses PH domains that appear to be highly specific for PtdIns(3,4,5) P_3 ; for example, the PH domain of GRP1 has an affinity for the Ins(1,3,4) P_3 headgroup configuration that is at least 100-fold weaker than that for the Ins(1,3,4,5) P_4 headgroup that occurs in PtdIns(3,4,5) P_3 [5]. These data, together with the observation that PtdIns(3,4) P_2 can be generated in the absence of a PtdIns(3,4,5) P_3 signal after activation and cross-linking of α ii/ β iii integrins in platelets, suggest that PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 can both function as second messengers with distinct, but overlapping, functions [3,4].

In order to evaluate the signalling roles of 3-phosphoinositides, reliable methods are required for the detection, localization and quantitative measurement of these lipids in stimulated cells. We developed a radioligand displacement assay that reliably detects PtdIns(3,4,5) P_3 [14], greatly simplifying the analysis of this lipid,

Abbreviations used: Btk, Bruton's tyrosine kinase; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GRP1, general receptor for phosphoinositides-1; PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PKB, protein kinase B; PH(GRP1)GFP and PH(PKB)GFP, PH domains of GRP1 and PKB respectively expressed as GFP fusion proteins; PI 3-kinase, phosphoinositide 3-kinase; rhodamine-PtdEtn, rhodamine-labelled phosphatidylethanolamine.

¹ To whom correspondence should be addressed (e-mail agray@bad.dundee.ac.uk).

but the only current method for the measurement of $\text{PtdIns}(3,4)P_2$ requires laborious cell labelling and chromatography procedures [3,4,15]. Moreover, these biochemical approaches are not well suited to determining the subcellular localization of lipid signals.

Recently, several groups have reported the use of fluorescence microscopy to monitor the cellular redistribution of suitably tagged and expressed PH domains, or PH-domain-containing proteins, in response to stimuli that affect the concentrations of phosphoinositides. Surprisingly, none of these reports showed comparable biochemical analyses of $\text{PtdIns}(3,4,5)P_3$ levels within the cells under study. More importantly, they did not attempt to distinguish between the accumulation of $\text{PtdIns}(3,4,5)P_3$ and $\text{PtdIns}(3,4)P_2$ [6,13,16]. We now compare two methods of 3-phosphoinositide analysis in HEK 293 cells responding to insulin: translocation of a fusion protein comprising green fluorescent protein (GFP) and the PH domain of PKB [PH(PKB)GFP] and biochemical analysis by a radioligand displacement assay. In addition, we show selective accumulation of $\text{PtdIns}(3,4)P_2$ in Swiss 3T3 cells subjected to an oxidative stress (1 mM H_2O_2), and use this system to evaluate the lipid-binding specificities of the PH domains of PKB and GRP1 *in vivo*. The results suggest that $\text{PtdIns}(3,4)P_2$ and $\text{PtdIns}(3,4,5)P_3$ can be distinguished in single cells, and further emphasize the distinct, but overlapping, signalling roles of these lipids.

MATERIALS AND METHODS

Construction of fusion proteins

The GFP-tagged N-terminal PH domain of PKB was constructed by subcloning, in frame, a 750 bp *Bam*HI/*Apa*I fragment, encoding the first 250 amino acids of human PKB α , into the *Bgl*II/*Apa*I sites of the GFP mammalian expression vectors pEGFP-N1 and pEGFP-C1 (Clontech). The two constructs produced encode the PKB PH domain fused to the C- and N-termini respectively of GFP. For transfection experiments, DNA was produced using a Qiagen Mega Prep kit and used without further purification. The PH domain of mouse GRP1 (amino acids 263–380 [6]), was cloned by PCR from a mouse brain cDNA library (Stratagene) using 5' oligo TGAAAGTATCAAGAATGAGCCG and 3' oligo CTTGGCTGGCTGTTCTTGA-GGG 100 bp 3' of the stop codon. The single 451 bp product was cloned into TOPO 2.1 (Invitrogen) and several clones were sequenced. An error-free product was subcloned in-frame into the EGFP C1 vector (Clontech) for use in transfection experiments.

Cell culture methods

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% (v/v) fetal calf serum (Sigma), as were Swiss 3T3 cells with the additional supplement of glutamine. Transfections were carried out using 1 μg of plasmid DNA and 3 μl of Eugene 6 liposomal transfection reagent (Boehringer) according to the manufacturer's instructions. Typically, 35 mm culture dishes of HEK 293 cells at 60–70% confluency containing 2 ml of complete DMEM were transfected as above. This routinely resulted in 40–60% transfection efficiency, as determined by visualization of GFP by fluorescence microscopy 24 h after transfection. In the case of Swiss 3T3 cells, the same protocol was employed, with the exception that the cells were grown on glass coverslips in 6-well plates. Under these conditions, 10–15% transfection efficiency was routinely obtained. The efficiency of expression of fusion protein varied by 2–3-fold between transfected cells in both cell types.

Microscopy methods

Confocal microscopy of living cells was carried out as follows. Cells were made quiescent by washing three times in serum-free DMEM and then incubation in DMEM supplemented with 0.5% (w/v) BSA at 37 °C in a CO_2 incubator for 3 h. Before use, cells were transferred into modified Krebs buffer (25 mM Hepes, pH 7.4, 118.4 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO_4 , 1.29 mM CaCl_2 , 1.18 mM K_2HPO_4 , 11.7 mM glucose) and viewed with a 40 $\times$ water-immersion objective. In dual-labelling experiments, cells were pre-incubated at 37 °C in a CO_2 incubator for 10 min in serum-free DMEM containing 2 $\mu\text{g}/\text{ml}$ (diluted from a 1 mg/ml sonicated vesicle stock) rhodamine-labelled phosphatidylethanolamine (rhodamine-PtdEtn; Avanti Lipids Ltd.). Cells were then washed three times in Krebs buffer before use. In a typical experiment, a group of transfected cells was selected at random and the confocal image adjusted to take a section through the body of the cell, including the nucleus. Additions were then made to the medium and images were collected at appropriate time intervals.

In experiments using Swiss 3T3 cells, the coverslips were incubated in modified Krebs buffer and allowed to equilibrate for 15 min before any additions. Incubations were terminated by aspiration of the Krebs buffer and addition of 3% paraformaldehyde in isotonic PBS. Cells were incubated for 15 min at room temperature in the paraformaldehyde/PBS fixative, before being washed three times in PBS and mounted onto slides for confocal microscopy.

Radioligand displacement assay

HEK 293 cells in 35 mm-diam. dishes were equilibrated in modified Krebs buffer and stimulated with insulin for various times or treated with wortmannin (100 nM) after insulin stimulation. The cells were incubated at room temperature to mimic the conditions used for microscopy of live cells, and incubations were terminated by removal of the medium and addition of 0.5 M trichloroacetic acid. The samples were then processed for determination of $\text{PtdIns}(3,4,5)P_3$ mass using an $\text{Ins}(1,3,4,5)P_4$ -specific radioligand displacement assay as described in [14].

Cell labelling and HPLC analysis of lipids

Swiss 3T3 mouse fibroblasts were seeded in 6-well plates at 10^4 cells/cm² in inositol-free DMEM supplemented with 10% (v/v) dialysed fetal bovine serum. After 48 h the medium was replaced with 4 ml of fresh medium containing 40 $\mu\text{Ci}/\text{ml}$ [³H]inositol and labelled for a further 48 h. The medium was aspirated and the cells were washed twice with 2 ml of modified Krebs buffer, and then conditioned for 45 min in 3 ml of the same buffer in a 37 °C water bath. Cells were treated as described above, but were quenched with 1 ml of ice-cold 10% (w/v) trichloroacetic acid, scraped from the plates, on ice, and transferred to Eppendorf tubes. The wells were rinsed once with 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid and the particulate material was pelleted by centrifugation (5 min at 20800 g). The supernatant was removed and the pellet was washed once with 1 ml of ice-cold 5% (w/v) trichloroacetic acid/1 mM EDTA. The lipids were extracted, on ice, for 20 min in 0.75 ml of chloroform/methanol/conc. HCl (40:80:1, by vol.). To obtain two phases, 0.25 ml of chloroform and 0.45 ml of 0.1 M HCl were added, and the samples were thoroughly vortex-mixed and spun for 2 min at 17500 g. The lower, organic phases were transferred to screw-cap tubes containing 50 μl of 1 M ammonia

in methanol. The upper phase and interphase were re-extracted once with 0.5 ml of synthetic lower phase and the organic phases were pooled, dried down in a vacuum concentrator and stored at -80°C .

Deacylation of extracted lipids was carried out as described in [15], and the resulting glycerophosphoinositol phosphates were analysed by HPLC using a Partisphere SAX column ($5\text{ }\mu\text{m}$ mesh size; $4.6\text{ mm} \times 250\text{ mm}$). The elution was started with 5 min of water at a flow rate of 1 ml/min, followed by a gradient from water to 1 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.8 with orthophosphoric acid) over 100 min with an increment of 1 %/min. Three fractions were collected per min. Scintillant (3 ml) was added to each fraction, and radioactivity was determined by scintillation counting.

RESULTS

Reversible, PI 3-kinase-dependent, translocation of PH(PKB)GFP to plasma membranes

Confocal fluorescence microscopy of PH(PKB)GFP-transfected HEK 293 cells revealed that the expressed protein was found almost exclusively within the cytosolic compartment, as reported previously for these and other cells [12,16]. As shown in Figure 1(A), a small, but reproducible, amount of the fusion protein was constitutively present at the plasma membrane of unstimulated cells. As this population was sensitive to low levels of wortmannin (see Figure 4), it probably reflects the basal level of $\text{PtdIns}(3,4,5)\text{P}_3$ in these cells. Stimulation with insulin (Figure 1B) caused a marked translocation of the fusion protein, with increased fluorescence being observed fairly uniformly at the cell periphery, presumably at the plasma membrane. Enhanced membrane fluorescence was observed within the first 1 min of stimulation

Figure 2 Reversal of insulin-induced PH(PKB)GFP binding to the plasma membrane by wortmannin in HEK 293 cells

Shown are confocal micrographs of the same cells that have been stimulated with insulin for 15 min (A) and then incubated for a further 15 min in the presence of 100 nM wortmannin (B). The micrographs are representative of four independent experiments.

and persisted for at least 60 min in the continued presence of insulin. HEK 293 cells were chosen to test this system because their PI 3-kinase response to insulin stimulation has been well

Figure 1 Localization of PH(PKB)GFP fusion protein in HEK 293 cells in response to insulin stimulation

Shown are confocal micrographs of the same cells before (A) and after (B) 15 min of insulin stimulation. The green image shows the GFP distribution, and the red image the distribution of membrane material marked by rhodamine-PtdEtn. Arrows mark points of interest described in the text. The data presented are representative of four independent experiments.

Figure 3 Quantitative analysis of the time course of redistribution of PH(PKB)GFP in response to insulin stimulation and its reversal by wortmannin in HEK 293 cells

Fluorescence intensity was measured as a function of distance across the cell after insulin stimulation at the time points shown (A). The measurements were then repeated at the times shown after the addition of 100 nM wortmannin (B). For clarity, only the start and end points of each experiment are shown. All data are normalized to mean cytoplasmic fluorescence, as described in the Materials and methods section.

characterized in our laboratory, and they are known to show relatively small changes in $\text{PtdIns}(3,4,5)\text{P}_3$ levels, providing a rigorous test of the system.

In experiments of this type, it is important to differentiate between true translocation and redistribution of material that may already be associated with the cellular structures being examined. This is particularly important in these experiments, since HEK 293 cells undergo a substantial shape change upon stimulation with insulin. In order to eliminate this possibility, the transfected cells were incubated with rhodamine-PtdEtn, which labels the bulk lipid phase of cellular membranes, before stimulation with insulin. This method has been used to good effect in previously published work [12]. The green-channel images in Figures 1(A) and 1(B) clearly demonstrate the shape change that HEK 293 cells undergo upon insulin stimulation. Membrane ruffles formed during this process tend to cause an increase in membrane density within the confocal image, and hence intensification of the signal from the rhodamine-PtdEtn as well as from the GFP reporter. However, the accumulation of GFP fusion protein upon insulin stimulation did not mirror the apparent redistribution of rhodamine-PtdEtn. This is indicated by the arrows in Figure 1(B), which highlight points of accumulation of the fusion protein (green channel) associated with reduced membrane density (red channel). The images in Figures 1(A) and

Figure 4 Correlation of redistribution of GFP-tagged PH domains with cellular levels of $\text{PtdIns}(3,4,5)\text{P}_3$ in HEK 293 cells

The peaks of fluorescence associated with the plasma membrane during insulin stimulation and subsequent wortmannin treatment, measured as in Figure 3, were integrated at the time points shown. $\text{PtdIns}(3,4,5)\text{P}_3$ mass (●; PIP_3) was estimated by the radioligand displacement assay (as described in the Materials and methods section) in identically treated cells. The $\text{PtdIns}(3,4,5)\text{P}_3$ mass data are presented as means $\pm$ S.D. of triplicate determinations from two independent experiments. The fluorescence data for the PKB PH domain (▲) and the GRP1 PH domain (■) are means $\pm$ S.D. of integrated fluorescence, normalized to mean cytoplasmic fluorescence, from left and right sides of three cells in two independent experiments. The arrow marks the point of addition of wortmannin (100 nM).

1(B) are in the same focal plane and differences are due to movement of the cells. The rhodamine image shows the rhodamine-PtdEtn labelling of intracellular membranes as well as the plasma membrane, most notably the nuclear membrane. Control cells transfected with native GFP showed no changes in GFP localization, apart from occasional spots apparently associated with membrane ruffles (results not shown).

The data presented were obtained using the PH domain of PKB fused to the N-terminus of GFP. Results obtained using a fusion protein in which the PH domain was fused to the C-terminus of GFP were essentially identical. Pretreatment of the cells with wortmannin (100 nM for 10 min) completely abolished any membrane localization of the GFP fusion protein and changes in cell shape in response to insulin stimulation (results not shown). The addition of wortmannin to cells prestimulated with insulin for 15 min reversed the targeting of the fusion protein to the plasma membrane with a half-time of approx. 2 min (see Figure 4). The results of a typical experiment, in which the depicted cells were stimulated with insulin for 15 min before the addition of 100 nM wortmannin for a further 15 min, are shown in Figure 2.

Quantitative analysis of PH(PKB)GFP binding to plasma membranes

In experiments similar to those described above, images of the cells were collected at various time points and the changes in fluorescence intensity, as a function of distance across individual cells, in response to insulin stimulation and subsequent treatment with wortmannin were quantified using Bio-Rad confocal image analysis software. Typical changes in fluorescence intensity measured across a single cell are shown in Figure 3. These data from a single cell are representative of several experiments of this type. The data were normalized to the mean cytoplasmic fluorescence as follows. Data were collected at various intervals, but only the start and end points are shown in Figure 3 for clarity. At each time point, the fluorescence intensity values (pixels) from

Table 1 3-Phosphoinositide levels in [^3H]inositol-labelled Swiss 3T3 mouse fibroblasts

The results are presented as means $\pm$ S.D. of triplicate determinations in three independent experiments. Cells were grown to confluence in 6-well plates and were labelled with $10\ \mu\text{Ci}/\text{ml}$ [^3H]inositol. After treatment at the indicated times, the cellular lipids were extracted and deacylated, and the resulting glycerophosphoinositide phosphates were analysed by HPLC.

Treatment	^3H (d.p.m.)		
	PtdIns3P	PtdIns(3,4) P_2	PtdIns(3,4,5) P_3
Control	15 902 $\pm$ 377	0	185 $\pm$ 94
H ₂ O ₂ (10 min)	10 506 $\pm$ 647	3610 $\pm$ 516	562 $\pm$ 209
H ₂ O ₂ (30 min)	11 596 $\pm$ 2027	3782 $\pm$ 217	187 $\pm$ 80
PDGF (10 min)	15 474 $\pm$ 303	1079 $\pm$ 138	1934 $\pm$ 38

the cytoplasm ($5\ \mu\text{m}$ in from the plasma membrane on each side of the cell) were averaged, and this value was subtracted from the values of all data points. This is essentially the method used by Stauffer et al. [13]. Figure 3(A) shows the redistribution of the GFP fusion protein to the edges of the cell over after a 15 min stimulation with insulin. Figure 3(B) shows the effect of the addition of 100 nM wortmannin for 15 min on the distribution

of the fusion protein in cells that had been stimulated with insulin for 15 min before the addition of wortmannin.

PKB PH domain translocation is correlated with PtdIns(3,4,5) P_3 production

In order to demonstrate that the observed localization in response to insulin and its reversal by wortmannin is likely to reflect changes in PtdIns(3,4,5) P_3 levels in the cell, the latter were measured directly using a recently developed radioligand displacement assay. Plates of cells were treated in exactly the same manner as those used in the microscopy experiments, except that the experiment was terminated by the addition of trichloroacetic acid at various time points and PtdIns(3,4,5) P_3 mass was determined. The PH domain of PKB is known to bind both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 ; therefore the quantification of membrane fluorescence was also carried out using a PH-(GRP1)GFP fusion protein, which is specific for PtdIns(3,4,5) P_3 , exactly as described for the PKB PH domain fusion protein. The data presented in Figure 4 show a clear correlation between the localization of the fusion protein to the plasma membrane and the amount of PtdIns(3,4,5) P_3 within the cell. The time course of the accumulation of PtdIns(3,4,5) P_3 (●) in response to insulin stimulation and its subsequent decline in response to the addition of the PI 3-kinase inhibitor wortmannin (as indicated by the

Figure 5 Swiss 3T3 cells transfected with PH(PKB)GFP

Panel 1: (A) unstimulated cells; (B) cells stimulated with 10 ng/ml PDGF for 10 min; (C), cells stimulated with 10 ng/ml PDGF for 30 min; (D) cells pretreated with 100 nM wortmannin for 10 min before stimulation with PDGF for 10 min. Panel 2 is as described for panel 1, except that cells were treated with 1 mM H₂O₂ instead of PDGF.

Figure 6 Swiss 3T3 cells transfected with PH(GRP1)GFP

Panel 1: (A) unstimulated cells; (B) cells stimulated with 10 ng/ml PDGF for 10 min; (C), cells stimulated with 10 ng/ml PDGF for 30 min; (D) cells pretreated with 100 nM wortmannin for 10 min before stimulation with PDGF for 10 min. Panel 2 is as described for panel 1, except that cells were treated with 1 mM H_2O_2 instead of PDGF.

arrow) is paralleled by the distribution of the fusion proteins PH(PKB)GFP (▲) and PH(GRP1)GFP (■) during insulin stimulation followed by wortmannin treatment.

Although the GFP fluorescence shows an excellent temporal correlation with the production of $PtdIns(3,4,5)P_3$, the magnitude of the fluorescence change does not show such a close correlation. There are two possible reasons for this. The PH domain of PKB could be detecting $PtdIns(3,4)P_2$ as well as $PtdIns(3,4,5)P_3$, resulting in a correspondingly larger signal than that detected by the $PtdIns(3,4,5)P_3$ -specific mass assay. This is supported by the fact that the GRP1 PH domain more closely reflects the changes in $PtdIns(3,4,5)P_3$ levels, especially the rapid fall following the addition of wortmannin. It should be noted that the fluorescence units are arbitrary, and the differences in fluorescence levels between the GRP1 and PKB PH fusion proteins simply reflects the difference in the intrinsic fluorescence of the two proteins. Alternatively, the discrepancy could be because the fluorescence data arise from measurements on a few cells, whereas the lipid mass determinations are carried out on a population of several million cells.

Differential detection of $PtdIns(3,4,5)P_3$ and $PtdIns(3,4)P_2$ by the PH domains of PKB and GRP1

Recent studies have identified a novel pathway for the synthesis of $PtdIns(3,4)P_2$ in fibrinogen-stimulated platelets which is in-

dependent of $PtdIns(3,4,5)P_3$ production. We have now observed a similar response in Swiss 3T3 cells subjected to oxidative stress by exposure to 1 mM H_2O_2 . The details of this response will be reported elsewhere, but the features exploited here are illustrated in Table 1. Analysis of [3H]inositol-labelled cells confirmed that platelet-derived growth factor (PDGF) induces a high level of PI 3-kinase activity, which results in large increases in the levels of both $PtdIns(3,4,5)P_3$ and $PtdIns(3,4)P_2$. In response to oxidative stress, however, there was only a small, transient, increase in $PtdIns(3,4,5)P_3$, but a large and sustained increase in $PtdIns(3,4)P_2$. Critically, after 30 min of stimulation, $PtdIns(3,4,5)P_3$ levels had returned to basal, whereas $PtdIns(3,4)P_2$ levels were still greatly enhanced. These observations in 3T3 cells provide an opportunity to test the ligand-binding specificity of the PH domains *in vivo*.

The confocal micrographs in Figure 5 show PH(PKB)GFP-transfected 3T3 cells treated with 10 ng/ml PDGF (row 1) or 1 mM H_2O_2 (row 2). Figure 5(A) shows unstimulated cells, Figure 5(B) cells after 10 min of stimulation, Figure 5(C) cells after 30 min of stimulation and Figure 5(D) cells pretreated for 5 min with 100 nM wortmannin before 10 min of stimulation. The arrows indicate the membrane localization of the GFP fusion protein in response to stimulation with either PDGF or H_2O_2 at both time points, which is completely abolished by pretreatment with wortmannin. In contrast, the data presented

in Figure 6, which were obtained from a repeat of the previous experiment using the PH(GRP1)GFP fusion protein in place of the PH(PKB)GFP fusion, show no membrane localization of the former at the 30 min time point after stimulation with H_2O_2 . This presumably reflects the specificity of the GRP1 PH domain for PtdIns(3,4,5) P_3 , whereas the PKB PH domain detects the PtdIns(3,4) P_2 present at this time point in response to oxidative stress (Table 1). These experiments were not conducted on single cells as was the case for insulin stimulation of HEK 293 cells, since exposure of GFP to UV light in the presence of an oxidizing agent results in instant and irreversible bleaching of the fluorescence.

DISCUSSION

The results show that a probe consisting of the PH domain of PKB fused to the N-terminus of GFP detects a signal generated at, or close to, the plasma membranes of HEK 293 cells stimulated with insulin, or of Swiss 3T3 cells stimulated with PDGF. It is likely that this signal comprises both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 , based on the following observations. First, the responses are blocked by inhibitors of PI 3-kinases and require only the phosphoinositide-binding PH domain, and not additional sequence elements of PKB (Figures 1 and 4). Similar results, using NIH 3T3 cells, were reported by Watton and Downward [16], who showed that PH domain mutations that prevented or reduced phosphoinositide binding prevented membrane translocation in response to stimulation of PI 3-kinase. Secondly, artefacts due to PI 3-kinase-induced changes in cell shape do not account for the enhanced membrane fluorescence, because a rhodamine-labelled phospholipid analogue, which acts as a marker of surface membranes, did not behave in the same way as the PH domain probe (Figure 1). Thirdly, the kinetics of membrane translocation precisely mirrored changes in the levels of PtdIns(3,4,5) P_3 measured using a specific radioligand displacement assay (Figure 4). Lastly, the retention of PH(PKB)-GFP at membranes in Swiss 3T3 cells stimulated with H_2O_2 for 30 min confirmed that this probe is also capable of interacting with PtdIns(3,4) P_2 *in vivo* (Figures 5 and 6).

The PH domains of PKB and GRP1 appear to be both sensitive and highly selective probes for cellular 3-phosphoinositides. For example, both must be capable of detecting PtdIns(3,4,5) P_3 /PtdIns(3,4) P_2 signals against a 1000-fold excess of PtdIns(4,5) P_2 under basal conditions. More significantly, they provide information on the spatial distribution of these membrane-associated second messengers. For example, recent work using a PKB PH domain construct similar to that described here has shown the localization of 3-phosphoinositides at sites of cell-cell and cell-matrix contacts in epithelial cells maintained in the absence of serum [16]. Although the fluorescence micrographs shown here for HEK 293 cells are compatible with general plasma membrane targeting in response to insulin, more highly resolving techniques, such as immuno-electron microscopy, will be needed to confirm the exact localization with respect to the plasma membrane. Such approaches will be particularly valuable in situations where it has been proposed that PI 3-kinase may be translocated to specific membrane sites. For example, in skeletal muscle and adipose tissue, it has been proposed that the distinctive effects of insulin on glucose uptake, mediated via translocation of the GLUT4 transporter to the plasma membrane, involve the targeting of PI 3-kinase to intracellular membranes rather than to the plasma membrane [17–20].

Several PH domains, including those found in ARNO (ADP-ribosylation factor nucleotide-binding site opener) [21], GRP1

[4], Btk (Bruton's tyrosine kinase) [22], phospholipase C- γ 1 [23] and PKB, have now been shown to be translocated to membranes upon activation of PI 3-kinases. Of these, the ligand-binding specificities of PKB, GRP1 and Btk have been best characterized *in vitro*, with the last two showing especially high specificity for PtdIns(3,4,5) P_3 , whereas PKB has been shown to bind both PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 . The observation that subjecting fibroblasts to an oxidative stress for 30 min caused a transient increase in PtdIns(3,4,5) P_3 and a sustained accumulation of PtdIns(3,4) P_2 (Table 1) allowed us to test this specificity in intact cells. Our results confirm that the PH domains of PKB and GRP1 detect distinct patterns of phosphoinositides in these cells (Figures 5 and 6). This dual binding specificity of PKB is likely to have functional consequences, since PKB appears to be activated in platelets by both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 [3,4].

GRP1 possesses a Sec7 homology domain which is required to stimulate guanine nucleotide exchange on ADP-ribosylation factors 1 and 5 *in vitro*, a reaction that is greatly stimulated by binding of PtdIns(3,4,5) P_3 to the PH domain [24,25]. The functions of GRP1, and other PtdIns(3,4,5) P_3 -specific binding proteins, would therefore be expected to be stimulated upon activation of type I PI 3-kinases, inhibited by the actions of PtdIns(3,4,5) P_3 5-phosphatases [which convert PtdIns(3,4,5) P_3 into PtdIns(3,4) P_2] and insensitive to stimuli that increase PtdIns(3,4) P_2 levels without affecting PtdIns(3,4,5) P_3 . By contrast, all three conditions would be expected to support PKB activation. In this regard, it is important to note that the PH domain of PDK1, the upstream kinase responsible for the phosphorylation-dependent activation of PKB, similarly binds both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 . Overall, our data support the argument that PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 are independent second messengers capable of directing distinct cellular responses through interactions with overlapping sets of PH-domain-containing proteins. It is of obvious importance, therefore, to develop methods capable of distinguishing these signalling molecules, especially within the cellular environment in which they act. The combined use of GFP-tagged PH domains of GRP1 and PKB described here is a first step in that direction. Further characterization of the ligand-binding specificities of other 3-phosphoinositide-binding PH domains, such as those of Vav [26], Sos [27] and Tiam-1 [28], may provide additional tools for this purpose. The identification of proteins that interact specifically with PtdIns(3,4) P_2 , but not PtdIns(3,4,5) P_3 , would be especially valuable.

One note of caution, however, arises from studies of the subcellular localization of PDK1, which is constitutively present both in the cytosol and at the plasma membrane, and which, despite the characteristics of its PH domain, does not appear to be translocated upon activation of PI 3-kinase [12] (but see [29] for an alternative view). It now seems likely that the subcellular distribution of PDK1 involves protein/protein as well as protein/lipid interactions [11]. For these reasons, isolated lipid-binding domains are likely to prove to be probes that are more useful for lipid second messengers than the intact proteins from which they are derived.

REFERENCES

- 1 Vanhaesebroeck, B., Leevers, S. J., Panayotou, G. and Waterfield, M. D. (1997) Trends Biochem. Sci. **22**, 267–272
- 2 Toker, A. and Cantley, L. C. (1997) Nature (London) **387**, 673–676
- 3 Banfic, H., Downes, C. P. and Rittenhouse, S. E. (1998) J. Biol. Chem. **273**, 11630–11637
- 4 Banfic, H., Tang, X., Batty, I. H., Downes, C. P., Chen, C. and Rittenhouse, S. E. (1998) J. Biol. Chem. **273**, 13–16

- 5 Duckworth, B. C. and Cantley, L. C. (1996) *Handb. Lipid Res.* **8**, 125–165
- 6 Venkateswarlu, K., Gunn-Moore, F., Oatey, P. B., Tavaré, J. M. and Cullen, P. J. (1998) *Biochem. J.* **335**, 139–146
- 7 Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997) *Curr. Biol.* **7**, 261–269
- 8 Meier, R., Alessi, D. R., Cron, P., Andjelkovic, M. and Hemmings, B. A. (1997) *J. Biol. Chem.* **272**, 30491–30497
- 9 Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M. and Hemmings, B. A. (1997) *J. Biol. Chem.* **272**, 31515–31524
- 10 Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) *Biochem. J.* **331**, 299–308
- 11 Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R. A., Downes, C. P. and Alessi, D. R. (1999) *Curr. Biol.* **9**, 393–404
- 12 Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R. and Lucocq, J. (1999) *Biochem. J.* **337**, 575–583
- 13 Stauffer, T. P., Seungkriol, A. and Meter, T. (1998) *Curr. Biol.* **8**, 343–346
- 14 Van der Kaay, J., Cullen, P. J. and Downes, C. P. (1997) in *Methods in Molecular Biology, Phospholipid Signalling Protocols* (Bird, I. M. and Totowa, N. J., eds.), pp. 109–125, Humana Press Inc., Clifton, NJ
- 15 Batty, I. H. and Downes, C. P. (1996) *Biochem. J.* **317**, 347–351
- 16 Watton, S. J. and Downward, J. (1999) *Curr. Biol.* **9**, 433–436
- 17 Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M. and Holman, G. D. (1994) *Biochem. J.* **300**, 631–635
- 18 McDowell, H. E., Walker, T., Hajdich, E., Christie, G., Batty, I. H., Downes, C. P. and Hundal, H. S. (1997) *Eur. J. Biochem.* **247**, 306–313
- 19 Kummer, J. L., Calera, M. R. and Pilch, P. F. (1998) *Biochem. Biophys. Res. Commun.* **246**, 282–286
- 20 Clark, S. F., Martin, S., Carozzi, A. J., Hill, M. M. and James, D. E. (1998) *J. Cell Biol.* **140**, 1211–1225
- 21 Venkateswarlu, K., Oatey, P. B., Tavaré, J. M. and Cullen, P. J. (1998) *Curr. Biol.* **8**, 463–466
- 22 Varnai, P., Rother, K. I. and Balla, T. (1999) *J. Biol. Chem.* **274**, 10983–10989
- 23 Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A. and Schlessinger, J. (1998) *EMBO J.* **17**, 414–422
- 24 Klarlund, J. K., Guilherme, A., Holik, J. J., Virbasius, J. V., Chawla, A. and Czech, M. P. (1997) *Science* **275**, 1927–1930
- 25 Klarlund, J. K., Rameh, L. E., Cantley, L. C., Buxton, J. M., Holik, J. J., Sakelis, C., Patki, V., Corvera, S. and Czech, M. P. (1998) *J. Biol. Chem.* **273**, 1859–1862
- 26 Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A. and Broek, D. (1998) *Science* **279**, 558–560
- 27 Nimmual, A. S., Yatsula, B. A. and Bar-Sagi, D. (1998) *Science* **279**, 560–563
- 28 Rameh, L. E., Arvidsson, A. K., Carraway, III, K. L., Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J. et al. (1997) *J. Biol. Chem.* **272**, 22059–22066
- 29 Anderson, K. E., Coadwell, J., Stephens, L. R. and Hawkins, P. T. (1998) *Curr. Biol.* **8**, 684–691

Received 21 June 1999/7 September 1999; accepted 23 September 1999