RESEARCH COMMUNICATION

Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation

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Recent evidence has suggested that activation of phosphoinositide 3-kinase (PI 3-kinase) is required for the activation of Akt-1 by growth factors and insulin. Here we demonstrate by two independent methods that Akt-1 from L6 myotubes binds to PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns(4,5)P_2 when presented against a background of phosphatidylserine (PtdSer) or a 1:1 mixture of PtdSer and phosphatidylcholine (PtdCho). No binding was observed with the lipids PtdIns(3,5)P_2, PtdIns4P and PtdIns3P or background lipids. Activated, hyperphosphorylated forms of Akt-1 from insulin-stimulated L6 myotubes bound to PtdIns(3,4,5)P_3 in a similar manner as inactive Akt-1. Quantitative analysis using surface plasmon resonance showed that the equilibrium association constant for the binding of Akt-1 to PtdIns(3,4,5)P_3 was submicromolar and that PtdIns(3,4)P_2 and PtdIns(4,5)P_2 bound to Akt-1 with 3- and 6-fold lower affinities respectively. Interaction of Akt-1 with PtdIns(3,4,5)P_3 did not activate the protein kinase activity, either before or after incubation with MgATP. A model is presented in which PtdIns(3,4,5)P_3 may prime Akt-1 for activation by another protein kinase, perhaps by recruiting it to the plasma membrane.

INTRODUCTION

Members of the family of phosphoinositide 3-kinases (PI 3-kinases) catalyse the phosphorylation of PtdIns(4,5)P_2, leading to the formation of the putative second messenger PtdIns(3,4,5)P_3 [1]. Although the direct physiological targets of PtdIns(3,4,5)P_3 are presently unknown, several lines of investigation have shown that PI 3-kinase lies upstream of several signalling protein kinase cascades. These include the activation of the p70 S6 kinase [2,3], activation of the p42/44 mitogen-activated protein kinase pathway by insulin, insulin-like growth factor-1 and interleukin-2 [4–6], and perhaps also the activation of the PAK protein kinases [7,8]. Most recently it has been shown that the serine/threonine protein kinase Akt-1 also lies downstream of PI 3-kinase [9–12].

Akt is expressed in all cells as three related isoforms [13–17], all of which possess an N-terminal pleckstrin homology domain of approx. 100 residues. Related pleckstrin homology domains are found in several other proteins involved in signal transduction whose function may be to interact with inositol phospholipids [18–20] and with G-protein $\beta\gamma$ subunits [21] or other effectors. Akt-1 is activated in cells by a variety of growth factors [9,10] as well as by insulin [11,22]. It becomes hyperphosphorylated when activated and can be inactivated *in vitro* by treatment with protein phosphatases that dephosphorylate serine and threonine residues [9–11,22], implying that Akt-1 is activated either by an autophosphorylation event, perhaps triggered by an interaction with PtdIns(3,4,5)P_3, or by a distinct protein kinase [23,24]. The first physiological substrate of Akt-1 was recently identified in L6 myotubes as glycogen synthase kinase-3 [22].

It has recently been shown, using a variety of techniques, that PI 3-kinase activity is required for activation of Akt-1 in cells [9–12,22]. Furthermore, addition of PtdIns3P to immunoprecipitates of Akt-1 was reported to elevate by up to 4-fold the kinase activity towards a non-physiological substrate, histone H2B [9]. However, the significance of these results is not clear, since stimulation of cells with many growth factors does not alter the intracellular levels of PtdIns3P and only the intracellular concentrations of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are increased [25]. We have therefore investigated whether Akt-1 from control and insulin-stimulated L6 myotubes can interact directly with PtdIns(3,4,5)P_3 and structurally related lipids and the effect such interactions have on Akt-1 kinase activity.

MATERIALS AND METHODS

Materials

Phosphatidylserine (PtdSer) was from Boehringer Mannheim and phosphatidylcholine (PtdCho) was from Sigma. PtdIns(4,5)P_2 and PtdIns4P were purified from Folch brain extract (Sigma) as previously described [26]. Di-palmitoyl PtdIns(3,4,5)P_3 was synthesized as described previously [27]. The dipalmitoyl analogues of PtdIns3P and PtdIns(3,5)P_2 were prepared using either DIBAL-H or trimethylaluminium to effect regioselective cleavage of *myo*-inositol orthoformate tribenzyl ether [28]. The resulting products were resolved with (*S*)-(−)-camphanic chloride and converted into the required PtdIns derivatives using P(III) coupling techniques. The dipalmitoyl analogue of PtdIns(3,4)P_2 was also produced from a DIBAL-H cleavage of a differentially protected *myo*-inositol orthoformate derivative, but resolution/protection was effected using (+)-camphor dimethyl acetal. Similar phosphitidylation strategies were followed as described above. All new compounds were characterized by spectroscopic (^{1}H- and ^{31}P-NMR and MS) techniques. Details of these syntheses will be published in the

Abbreviations used: BIA, biospecific interaction analysis; PI 3-kinase, phosphoinositide 3-kinase; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine, RU, resonance units; spr, surface plasmon resonance.

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near future. Specific anti-Akt-1 antibodies were raised in sheep to a peptide (FPQFSYSASSTA) corresponding to the C-terminal 12 residues of Akt-1, cross-linked to BSA using glutaraldehyde, and affinity purified using a peptide affinity column as described previously [29].

Preparation of L6 cytosol

L6 cells were differentiated into myotubes on 10-cm-diam. dishes as described previously [30]. Cells were starved overnight in Dulbecco's minimal essential medium in the absence of serum, and were incubated for 5 min at 37 °C with either buffer or 100 nM insulin. Cells were washed twice with ice-cold buffer containing 20 mM Hepes/NaOH, pH 7.4, 0.14 M NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$ and 25 mM glucose. Cell lysates were prepared in the absence of detergent by scraping cells from each dish into 0.5 ml of ice-cold buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol, 2 μM microcystin-LR, 0.2 mM PMSF, 1 mM benzamidine and 10 μg/ml leupeptin. The cells were then broken using a cell cracker in which the space between the ball bearing and the wall of the chamber was 8 μm. The cell lysate was centrifuged for 5 min at 1000 ***g***, and the supernatant was then spun for 30 min at 200000 ***g*** at 2 °C. The supernatant, termed L6 cytosol, was used in subsequent experiments.

Vesicle binding of Akt-1

Sucrose-loaded large unilamellar vesicles were prepared by extrusion through polycarbonate membranes, with pores of 100 nm diam., using a phospholipid extruder (Lipex Biomembranes) as described previously [31]. L6 cytosol (0.2 ml; protein concentration 0.5 mg/ml) was incubated with phospholipid vesicles for 10 min on ice followed by centrifugation at 140000 ***g*** for 30 min at 2 °C. Supernatants and vesicle pellets were separated; the pellets were washed twice with 50 mM Tris/HCl (pH 7.5)/150 mM NaCl and then resuspended in this buffer to 0.2 ml. The pellet and the supernatant fractions (20 μl each) were run on a 10 % polyacrylamide gel and then transferred for a total of 300 V h to a nitrocellulose membrane. Immunoblotting was carried out by incubating the blot with 0.2 μg/ml affinity-purified sheep anti-Akt-1 antibody, in 50 mM Tris (pH 7.5)/0.15 M NaCl/0.1 % (w/v) Tween/5 % (w/v) skimmed milk for 2 h at 20 °C. Detection of the Akt-1 band was carried out using standard protocols with an anti-sheep secondary antibody (Pierce) coupled covalently to horseradish peroxidase (diluted 1:5000), and detection was carried out using the Enhanced Chemiluminescence method (Amersham).

Measurement of lipid association by biospecific interaction analysis (BIA)

Examination of lipid binding to Akt-1 was performed by surface plasmon resonance (spr) using the BIAlite biosensor (Pharmacia Biosensor). BIA allows optical measurement of intermolecular interactions by immobilization of one molecular species at a gold-coated surface followed by introduction of a second molecule as an injected analyte. Specific interactions are detected by changes in the resonance of the plasmon electrons associated with the gold film [32]. Anti-Akt-1 antibody (70 μl; 5 μg/ml) was immobilized via free amino groups to a CM5 sensorchip, pretreated with 35 μl of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-[3-(diethylamino)propyl] carbodiimide (1:1 mixture) to coat it with imide groups. Unreacted imides were quenched with 35 μl of 1 M ethanolamine, and Akt-1 was captured by the antibody by injection of 30 μl of L6 cell lysate. Two buffer systems were used to measure phospholipid interactions with captured Akt-1. The first used commercial BIA buffer [20 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005 % (v/v) P20 detergent] to which was added 3.4 mM $MgCl_2$ and 3.4 mM $CaCl_2$. The second used a more 'physiological' buffer (buffer B) comprising 20 mM Hepes, pH 7.2, 120 mM KCl, 20 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$ and sufficient $CaCl_2$ for a free Ca^{2+} concentration of 200 nM (calculated using a K_s of EGTA for Ca^{2+} ions of 5.17×10^6 M^{-1}). Phospholipid vesicles, which were prepared by sonication of dried films into 10 mM Hepes/NaOH, pH 7.2, as 20-fold-concentrated stocks, were diluted in the appropriate flow-through buffer and injected into the biosensor. Association and dissociation times were 420 s and at least 400 s respectively. All experiments were performed at a flow rate of 10 μl/min and at 25 °C. The antibody surface was regenerated free of protein between sensorgrams by injection of 10 μl of 50 mM 3-cyclohexylamino 1-propanesulphonic acid (pH 11.6)/150 mM NaCl. Two controls were performed for all sensorgrams. Firstly, vesicle binding to the anti-Akt-1 antibody in the absence of Akt-1 was determined, and was $\leqslant 8$ % of Akt-1-specific binding. Secondly, a different sensorchip was used to immobilize pre-immune sheep Ig, and vesicle binding after injection of L6 cytosol was measured. Binding in this case saturated very rapidly and was $\leqslant 15$ % of Akt-1-specific binding and probably represents binding to non-specifically captured protein.

Measurement of Akt-1 protein kinase activity

Akt-1 was immunoprecipitated from L6 cytosol by incubation at 2 °C with 2 μg of affinity-purified anti-Akt-1 antibody conjugated to 5 μl of Protein G–Sepharose. Immunoprecipitates were washed three times with 1.0 ml of 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1 mM EDTA/0.1 % (v/v) 2-mercaptoethanol (Buffer A) containing 0.5 M NaCl and washed twice with 1.0 ml of Buffer A and assayed for kinase activity using the peptide termed Crosstide (GRPRTSSFAEG) as substrate [22].

RESULTS

Akt-1 binds specifically to PtdIns(3,4,5)P_3-containing unilamellar vesicles

We investigated whether Akt-1 is able to associate directly with phospholipids by incubating cell lysates with sucrose-loaded vesicles of different compositions, followed by ultracentrifugation to separate free protein from lipid-bound protein. Two mole fractions of phosphoinositide in the vesicles were used (0.09 or 0.009) against a background of PtdSer or a 1:1 mixture of PtdSer/PtdCho. These lipids were chosen because both served to dilute the high charge of phosphoinositides in bilayers, thus reducing electrostatic effects, and because PtdSer facilitates the association of some proteins with lipid interfaces [31,33]. L6 cytosol was incubated with bulk phosphoinositide concentrations of 25 or 50 μM, and the vesicles were then pelleted by centrifugation and washed. At low concentrations of phosphoinositide (1 μM), no binding to any vesicle population was observed, possibly due to lipid metabolism. Anti-Akt-1 immunoblots of supernatants and vesicle pellets (Figure 1, upper panel) showed that for L6 cytosol incubated with PtdSer vesicles containing PtdIns(3,4,5)P_3 at a mole fraction of 0.09, about 90 % of the immunoreactive Akt-1 interacted with the vesicles. In parallel experiments using vesicles containing PtdIns(3,4)P_2 or PtdIns(4,5)P_2, about 50 % and 10 % of the cytosolic Akt-1 was bound respectively. In contrast Akt-1 did not bind to vesicles containing PtdIns(3,5)P_2, PtdIns3*P* or PtdSer alone. Similar data were obtained when L6 cytosol was incubated with phospho-

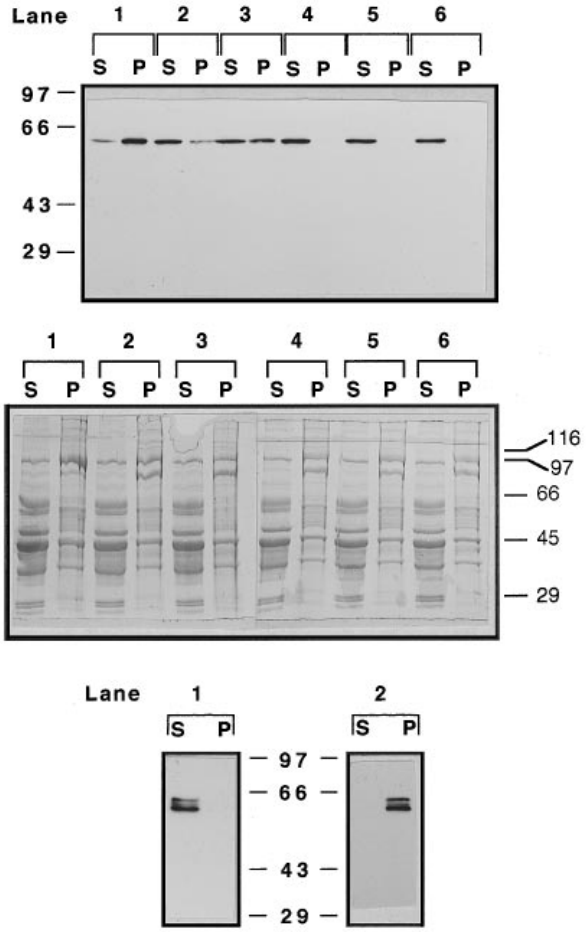

Figure 1 The protein kinase Akt-1 binds to phospholipid vesicles containing phosphatidylinositol bis- and tris-phosphates

Upper panel: L6 cytosol derived from unstimulated cells was incubated for 10 min on ice with sucrose-loaded vesicles containing PtdSer and the indicated phosphoinositides in molar ratios of 10:1. Bulk phosphoinositide concentrations were 50 μM and the mole fraction was 0.09. Lipid-bound and free protein were separated by centrifugation as described in the Materials and methods section, and supernatant (S) and pellet (P) fractions were immunoblotted using anti-Akt-1 antibodies. Lanes are: 1, PtdSer + PtdIns(3,4,5)P_3; 2, PtdSer + PtdIns(4,5)P_2; 3, PtdSer + PtdIns(3,4)P_2; 4, PtdSer + PtdIns(3,5)P_2; 5, PtdSer + PtdIns3P; 6, PtdSer alone. Middle panel: As for the upper panel except that L6 cytosol was incubated with vesicles containing PtdCho, PtdSer and phosphoinositides (10:10:1 by molarity). Gels stained with Coomassie Brilliant Blue are shown, emphasizing the non-specific binding of cytosolic protein to lipid. Phosphoinositide contents of the different lanes are: 1, PtdIns(3,4,5)P_3; 2, PtdIns(3,4)P_2; 3, PtdIns(3,5)P_2; 4, PtdIns(4,5)P_2; 5, PtdIns3P; 6, no phosphoinositide. Molecular-mass markers are indicated in kDa. Lower panel: Same as for the upper panel, except that L6 myotubes were stimulated with insulin for 5 min before lysis. Lanes are: 1, PtdSer/PtdCho (1:1) alone; 2, PtdSer/PtdCho + PtdIns(3,4,5)P_3 (10:10:1 by molarity). The Figure shows representative results and similar data were obtained in three experiments with each phospholipid in the upper panel and two experiments with each phospholipid in the lower panel.

inositides at a mole fraction of 0.009, diluted with a 1:1 mixture of PtdSer and PtdCho (results not shown). In contrast to the specific behaviour of Akt-1, similar ranges of proteins bound non-specifically and with low affinity to all vesicle preparations regardless of their phosphoinositide content, revealed by Coomassie Brilliant Blue staining of SDS gels (Figure 1, middle panel).

Figure 1 (lower panel) shows that activated Akt-1 from insulin-stimulated cells behaved similarly to inactive protein from control cells. Activated hyperphosphorylated Akt-1 bound to Ptd Ins(3,4,5)P_3-containing vesicles in a similar manner to the inactive dephosphorylated enzyme. No interaction was observed between any of the hyperphosphorylated species of Akt-1 and control vesicles containing only 1:1 mixture of PtdSer/PtdCho.

Quantitative measurement of Akt-1 binding to lipid vesicles using spr

Further characterization of the interaction between Akt-1 and phosphoinositide lipids was made using the BIAlite biosensor, a technique that permitted detection and kinetic analysis of interactions between Akt-1 and lipid vesicles (see the Materials and methods section). The rationale behind these experiments was that, insofar as Akt-1 does not bind to PtdSer or PtdCho, any binding of mixed composition vesicles observed with the BIAlite would be due to interaction with the phosphoinositide component, permitting quantification of the binding constants with respect to the phosphoinositide bulk concentration.

Figure 2 shows sensorgrams of binding of phosphatidylinositol bisphosphates and phosphatidylinositol trisphosphate to Akt-1 in the BIA buffer system. Akt-1 bound four times more PtdIns(3,4,5)P_3 than PtdIns(3,4)P_2 and up to 10 times more PtdIns(3,4,5)P_3 than PtdIns(4,5)P_2. In contrast, no significant binding of PtdIns(3,5)P_2 (Figure 2) and PtdSer/PtdCho (results not shown) was observed above controls. Sensorgram data were fitted to a homogeneous binding model from which values for association and dissociation rate constants were derived for the two buffer systems used (Table 1). The affinity of Akt-1 for PtdIns(3,4,5)P_3 was 3-fold greater than for PtdIns(3,4)P_2 and 6–7-fold greater than its affinity for PtdIns(4,5)P_2. Although it is formally possible that vesicle binding to other proteins captured by the immunoglobulin contributed to the binding observed, these data are consistent with Figure 1, representing binding predominantly to Akt-1.

Effect of PtdIns(3,4,5)P_3 on the protein kinase activity of Akt-1

Stimulation of L6 myotubes with insulin resulted in a maximal (10-fold) activation of Akt-1 within 5 min [22]. In order to investigate whether Akt-1 from unstimulated cells could autoactivate in the presence of PtdIns(3,4,5)P_3, Akt-1 immunoprecipitated from L6 cytosol was incubated with various phosphoinositide/PtdSer/PtdCho lipid mixtures in the presence of MgATP for 10 min and assayed for Crosstide kinase activity (Table 2). In these experiments different combinations of PtdIns(3,4,5)P_3 or other phosphoinositides failed to cause a significant increase in the basal Akt-1 kinase activity associated with the immunoprecipitates. In parallel experiments the activity of Akt-1 from insulin-stimulated cells was also unaffected by inositol phospholipids. Since the failure to observe activation of Akt-1 might be due to an inhibitory effect caused by the interaction of the antibody with Akt-1, we repeated these experiments using Akt-1 that had been pelleted from the cytosol of unstimulated L6 myotubes using PtdIns(3,4,5)P_3-containing vesicles. The vesicles were incubated for 10 min at 30 °C in the presence or absence of MgATP, then solubilized in buffer containing 1% (w/v) Triton X-100, and Akt-1 was immunoprecipitated and assayed. No significant activation of basal Akt-1 activity was detected in these experiments either (results not shown). These data therefore suggested that, in addition to no phosphoinositide-mediated change in Akt-1 kinase activity towards peptide substrate, Akt-1 did not autoactivate in the presence of PtdIns(3,4,5)P_3 under the conditions employed.

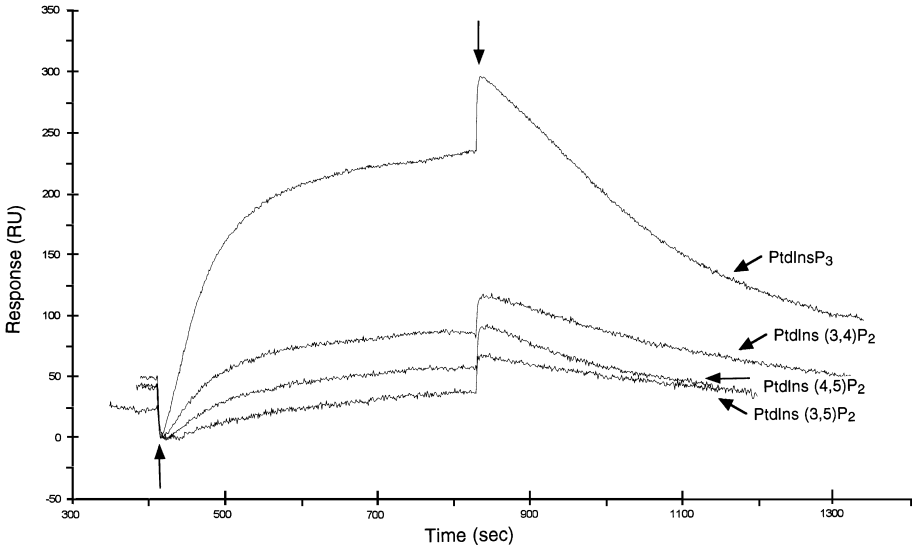

Figure 2 BIA sensorgrams demonstrating greater binding of Akt-1 to PtdIns(3,4,5)P_3 than to phosphatidylinositol bisphosphates

The Akt-1 antibody was immobilized to a sensorchip and used to capture Akt-1 from L6 cytosol. The upward arrow shows the point at which phospholipid vesicles (10 μM PtdCho/10 μM PtdSer/1 μM phosphoinositide) were injected, and the downward arrow shows the point at which dissociation started. All experiments were performed at 25 °C. Data were analysed using BIAevaluation software version 2.1, and the Figure shows representative traces of multiple sensorgrams for each phosphoinositide. Injection of different lipids caused the bulk refractive index to drop immediately before the association phases. Each sensorgram has therefore been normalized to zero at the minimum absorbance for each trace, to facilitate comparison between the different lipids. RU, resonance unit.

Table 1 Equilibrium association constants for binding of phosphoinositide-containing vesicles to Akt-1

Akt-1 was captured at a sensorchip surface as described in the Materials and methods section, and binding of mixed composition phosphoinositide-containing vesicles was measured by spr. Experiments were performed in two buffer systems, and K_a values for both are shown. Values are means ± S.D. for at least six determinations from at least three different sensorgrams, except for PtdIns(4,5)P_2, where values are from two sensorgrams.

	K_a (M^{-1})	
Phosphoinositide	BIA buffer	Buffer B
PtdIns(3,4,5)P_3	$4.28 \pm 0.9 \times 10^6$	$1.54 \pm 0.44 \times 10^8$
PtdIns(3,4)P_2	$1.84 \pm 1.5 \times 10^6$	$5.0 \pm 3.9 \times 10^7$
PtdIns(4,5)P_2	$0.72 \pm 0.04 \times 10^6$	$3.0 \pm 0.1 \times 10^7$
PtdIns(3,5)P_2	did not bind above controls	

Table 2 Akt-1 does not autoactivate in the presence of MgATP and PtdIns(3,4,5)P_3

Akt-1 was immunoprecipitated from L6 cytosol (0.2 mg of protein) from either control or insulin-stimulated cells in the absence of detergents. The immunoprecipitates were washed extensively in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1% (v/v) 2-mercaptoethanol and incubated with either buffer or 10 μM PtdSer containing 1 μM phosphoinositide on a shaking platform at 30 °C in the presence of 10 mM magnesium acetate/0.1 mM [γ-^{32}P]ATP. After 10 min, Crosstide substrate was added and Akt-1 activity was measured as described previously [22]. The data are presented for each lipid relative to those obtained for Akt-1 immunoprecipitated from unstimulated cells and incubated with buffer in the absence of lipid. The results given are means ± S.E.M. for three separate experiments. Akt-1 immunoprecipitated from unstimulated cells assayed in the absence of lipid had an activity of 7 ± 2 units/mg against Crosstide. nd, not determined.

	Relative activity of Akt-1 immunoprecipitates	
Lipid	Control cells	Insulin-stimulated cells
Buffer	1.0	11
PtdSer	1.1 ± 0.1	10 ± 3
PtdSer + PtdIns(3,4,5)P_3	1.2 ± 0.2	9 ± 2
PtdSer + PtdIns(4,5)P_2	1.0 ± 0.1	10 ± 2
PtdSer + PtdIns(3,4)P_2	1.2 ± 0.2	nd
PtdSer + PtdIns(3,5)P_2	0.9 ± 0.1	nd
PtdSer/PtdCho + PtdIns(3,4,5)P_3	0.70 ± 0.3	nd

DISCUSSION

The data presented here show that there is a relatively specific association between Akt-1 and PtdIns(3,4,5)P_3 *in vitro*. Akt-1 bound PtdIns(3,4,5)P_3 three times more avidly than PtdIns(3,4)P_2 and six times more strongly than PtdIns(4,5)P_2, and did not interact at all with PtdIns(3,5)P_2, PtdIns3*P*, PtdSer or PtdCho (Figures 1 and 2). It is not clear from our data whether the observed differences in binding affinities of Akt-1 to these different phospholipids are large enough to produce physiologically distinct effects. Stimulated concentrations of PtdIns(3,4,5)P_3 relative to PtdIns(4,5)P_2 are low (approx. 1%) and the 10-fold preference of Akt-1 for PtdIns(3,4,5)P_3 would therefore have to be accentuated in a cellular environment. There are several potential mechanisms by which this may occur, such as localized synthesis of PtdIns(3,4,5)P_3 or association of PtdIns(3,4,5)P_3 with a specific protein receptor at the plasma membrane of the cell, whose function when bound to PtdIns(3,4,5)P_3 might be to increase the affinity/specificity of Akt-1 for PtdIns(3,4,5)P_3. In this regard it should be noted that certain Src homology 2 domains may play such a role by being able to interact specifically with PtdIns(3,4,5)P_3 [34]. It is also conceivable that an unknown effector molecule could interact with Akt-1 and increase its affinity for PtdIns(3,4,5)P_3.

Apart from the Src homology 2 domains present on the p85 regulatory subunit of PI 3-kinase [34], Akt-1 is the only other protein with which PtdIns(3,4,5)P_3 has been shown to interact specifically to date. Other proteins that have been shown to

interact with PtdIns(3,4,5)P_3 are in the protein kinase C family, namely ϵ, δ, η, ζ and the protein kinase C-related kinase PRK1. However, the physiological relevance of this interaction is uncertain, since these enzymes also interact with PtdIns(4,5)P_2 with identical affinity [35–37]. The affinity of Akt-1 for phosphoinositides was markedly affected by the buffer composition used in the assay. Affinities were increased 30–40-fold when measured in a buffer containing 200 nM free Ca^{2+} (buffer B) compared with BIA buffer in which the free Ca^{2+} concentration was in the millimolar range. It is possible that binding of Akt-1 to PtdIns(3,4,5)P_3 is affected by Ca^{2+} ions, and since many stimuli that activated Akt-1 also activate phospholipase Cγ, it may be that the release of stored Ca^{2+} has a regulatory influence on the interaction of Akt-1 with the membrane.

Under the conditions used in this study, Akt-1 did not become active when associated with PtdIns(3,4,5)P_3-containing lipid vesicles alone, even after incubation with MgATP to see if PtdIns(3,4,5)P_3 might permit autophosphorylation and reactivation. Our data are in disagreement with those of Franke et al. [10], who found that addition of a mixture of non-purified enzymically prepared PtdIns3P of unknown concentration resulted in up to a 4-fold increase in the activity of Akt-1 immunoprecipitates towards histone 2B. Using two independent methods we found that Akt-1 did not interact with pure synthetic PtdIns3P in a PtdSer-containing vesicle under conditions in which $> 90\%$ of Akt-1 interacted with PtdIns(3,4,5)P_3-containing vesicles. We also failed to observe any activation of Akt-1 kinase activity when incubated with PtdIns3P (Table 1), even when using the same immunoprecipitation and assay conditions as used by Franke et al. [10] (results not shown).

The results presented in this paper suggest that some other protein/factor may therefore be required for phosphorylation and activation of Akt-1 *in vivo*. One possibility is that the generation of PtdIns(3,4,5)P_3 at the membrane of the cell may recruit Akt-1 from the cytosol to the plasma membrane where it is then activated by another kinase [23,24]. In this case, the role of PtdIns(3,4,5)P_3 would not be to activate Akt-1 but to prime it for activation. This would be analogous to the mechanism by which Ras is thought to prime c-Raf for activation in the classical MAP kinase pathway [38,39]. Furthermore, the oncogenic form of Akt-1 (v-Akt-1) expressed in the rodent acutely transforming retrovirus Akt8 is also anchored to the plasma membrane of cells, since it is expressed as a fusion protein with the membrane-associated GAG viral protein [15,40], and it may be this forced location in the cell that makes the v-Akt-1 oncoprotein constitutively active and thus results in cellular transformation.

In summary we have shown by two independent methods that the protein kinase Akt-1 binds to lipid interfaces containing PtdIns(3,4,5)P_3, PtdIns(3,4,)P_2 and PtdIns(4,5)P_2, but is not activated when located at such interfaces. Akt-1 has greater specificity for phosphatidylinositol trisphosphate than for any bisphosphate, and binding requires vicinal phosphate groups on the inositol head group. Identification of protein kinases that mediate the activation of Akt-1 and elucidation of the role of PI 3-kinase in this process are the key questions which must be addressed in future work.

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