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)\textsubscript{P}\textsubscript{3}, PtdIns(3,4)\textsubscript{P}\textsubscript{2} and PtdIns(4,5)\textsubscript{P}\textsubscript{2} when presented against a background of phosphatidylinerine (PtdSer) or a 1:1 mixture of PtdSer and phosphatidylcholine (PtdCho). No binding was observed with the lipids PtdIns(3,5)\textsubscript{P}\textsubscript{3}, PtdIns4P and PtdIns3P or background lipids. Activated, hyperphosphorylated forms of Akt-1 from insulin-stimulated L6 myotubes bound to PtdIns(3,4,5)\textsubscript{P}\textsubscript{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)\textsubscript{P}\textsubscript{3} was submicromolar and that PtdIns(3,4)\textsubscript{P}\textsubscript{3} and PtdIns(4,5)\textsubscript{P}\textsubscript{2} bound to Akt-1 with 3- and 6-fold lower affinities respectively. Interaction of Akt-1 with PtdIns(3,4,5)\textsubscript{P}\textsubscript{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)\textsubscript{P}\textsubscript{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)\textsubscript{P}\textsubscript{3}, leading to the formation of the putative second messenger PtdIns(3,4,5)\textsubscript{P}\textsubscript{3} \cite{1}. Although the direct physiological targets of PtdIns(3,4,5)\textsubscript{P}\textsubscript{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 \cite{2,3}, activation of the p42/44 mitogen-activated protein kinase pathway by insulin, insulin-like growth factor-1 and interleukin-2 \cite{4–6}, and perhaps also the activation of the PAK protein kinases \cite{7,8}. Most recently it has been shown that the serine/threonine protein kinase Akt-1 also lies downstream of PI 3-kinase \cite{9–12}.

Akt is expressed in all cells as three related isoforms \cite{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 \cite{18–20} and with G-protein βγ subunits \cite{21} or other effectors. Akt-1 is activated in cells by a variety of growth factors \cite{9,10} as well as by insulin \cite{11,22}. It becomes hyperphosphorylated when activated and can be inactivated \textit{in vitro} by treatment with protein phosphatases that dephosphorylate serine and threonine residues \cite{9–11,22}, implying that Akt-1 is activated either by an autophosphorylation event, perhaps triggered by an interaction with PtdIns(3,4,5)\textsubscript{P}\textsubscript{3} or by a distinct protein kinase \cite{23,24}. The first physiological substrate of Akt-1 was recently identified in L6 myotubes as glycogen synthase kinase-3 \cite{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 \cite{9–12,22}. Furthermore, addition of PtdIns3P to immuno-precipitates of Akt-1 was reported to elevate by up to 4-fold the kinase activity towards a non-physiological substrate, histone H2B \cite{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)\textsubscript{P}\textsubscript{3} and PtdIns(3,4)\textsubscript{P}\textsubscript{2} are increased \cite{25}. We have therefore investigated whether Akt-1 from control and insulin-stimulated L6 myotubes can interact directly with PtdIns(3,4,5)\textsubscript{P}\textsubscript{3} and structurally related lipids and the effect such interactions have on Akt-1 kinase activity.

MATERIALS AND METHODS
Materials
Phosphatidylinerine (PtdSer) was from Boehringer Mannheim and phosphatidylcholine (PtdCho) was from Sigma. PtdIns(4,5)\textsubscript{P}\textsubscript{3} and PtdIns4P were purified from Folch brain extract (Sigma) as previously described \cite{26}. Di-palmitoyl PtdIns(3,4,5)\textsubscript{P}\textsubscript{3} was synthesized as described previously \cite{27}. The dipalmitoyl analogues of PtdIns3P and PtdIns(3,5)\textsubscript{P}\textsubscript{2} were prepared using either DIBAL-H or trimethylaluminium to effect regioselective cleavage of myo-inositol orthoformate tribenzyl ether \cite{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 PtdIns3P and PtdIns(3,4)\textsubscript{P}\textsubscript{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 phosphitylation strategies were followed as described above. All new compounds were characterized by spectroscopic (\textsuperscript{1}H- and \textsuperscript{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, phosphatidylinerine, 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 MgSO₄, 1 mM CaCl₂, 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 20000 g 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 0.1% (w/v) 2-mercaptoethanol (Buffer 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, pre-treated 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 MgCl₂ and 3.4 mM CaCl₂. 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 MgCl₂ and sufficient CaCl₂ for a free Ca²⁺ concentration of 200 nM (calculated using a K° of EGTA for Ca²⁺ ions of 5.17 × 10⁻¹⁰ M⁻¹). Phospholipid vesicles, which were prepared by sonicating 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-propanesulfonic 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 ≤ 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 ≤ 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% (w/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 Crosseptide (GRPRTSSFAEG) as substrate [22].

RESULTS

Akt-1 binds specifically to PtdIns(3,4,5)P₃-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)P₃ 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)P₃ or PtdIns(4,5)P₃, 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)P₃, PtdIns3P, or PtdSer alone. Similar data were obtained when L6 cytosol was incubated with phospho-
cells. Activated hyperphosphorylated Akt-1 bound to PtdIns(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 auto-activate 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.
logically distinct effects. Stimulated concentrations of different phospholipids are large enough to produce physio-
observed differences in binding affinities of Akt-1 to these (Figures 1 and 2). It is not clear from our data whether the interact at all with PtdIns(3,5)
therefore have to be accentuated in a cellular environment. There
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except for PtdIns(4,5)
are means

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.

**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$_3$ and six times more strongly than PtdIns(4,5)P$_2$, and did not interact at all with PtdIns(3,5)P$_2$, PtdIns3P, 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 mM 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 C$_{y}$, 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 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 C$_{y}$, it may be that the release of stored Ca$^{2+}$ has a regulatory influence on the interaction of Akt-1 with the membrane.

The results presented in this paper suggest that some other protein/factor may therefore be required for phosphorylation and activation of Akt-1 in vitro. 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 oncogenic form of Akt-1 (v-Akt-1) expressed in the rodent

**REFERENCES**