Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα

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Background: Protein kinase B (PKB), also known as c-Akt, is activated rapidly when mammalian cells are stimulated with insulin and growth factors, and much of the current interest in this enzyme stems from the observation that it lies 'downstream' of phosphoinositide 3-kinase on intracellular signalling pathways. We recently showed that insulin or insulin-like growth factor 1 induce the phosphorylation of PKB at two residues, Thr308 and Ser473. The phosphorylation of both residues is required for maximal activation of PKB. The kinases that phosphorylate PKB are, however, unknown.

Results: We have purified 500 000-fold from rabbit skeletal muscle extracts a protein kinase which phosphorylates PKBα at Thr308 and increases its activity over 30-fold. We tested the kinase in the presence of several inositol phospholipids and found that only low micromolar concentrations of the D enantiomers of either phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) or PtdIns(3,4)P_2 were effective in potently activating the kinase, which has been named PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK1). None of the inositol phospholipids tested activated or inhibited PKBα or induced its phosphorylation under the conditions used. PDK1 activity was not affected by wortmannin, indicating that it is not likely to be a member of the phosphoinositide 3-kinase family.

Conclusions: PDK1 is likely to be one of the protein kinases that mediate the activation of PKB by insulin and growth factors. PDK1 may, therefore, play a key role in mediating many of the actions of the second messenger(s) PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2.

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Received: 18 February 1997
Revised: 4 March 1997
Accepted: 4 March 1997

Published: 19 March 1997
Electronic identifier: 0960-9822-007-00261

Current Biology 1997, 7:261–269

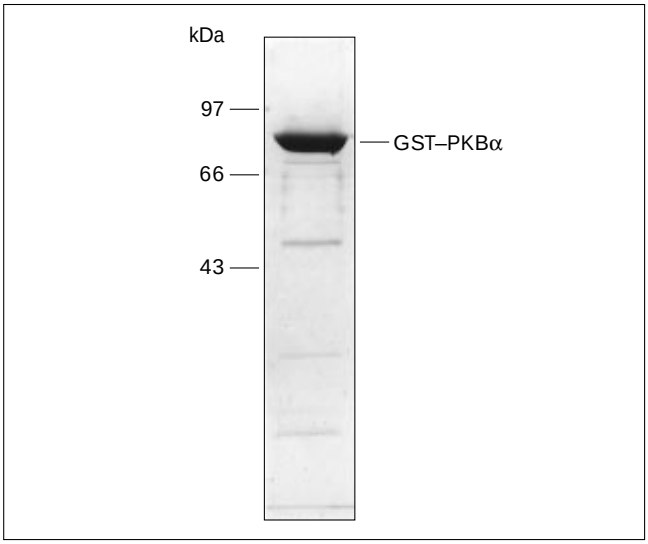
Background

Protein kinase B (PKB) [1], or RAC protein kinase [2], is the cellular homologue of a viral oncogene v-Akt [3] and has therefore also been termed c-Akt. The current interest in PKB stems firstly from the discovery that it is activated rapidly in response to insulin and growth factors and that this activation is prevented by inhibitors of phosphoinositide (PI) 3-kinase [4–6], and secondly from the finding that PKB isoforms are overexpressed in a significant percentage of ovarian, pancreatic [7,8] and breast cancer cells [2].

PKB appears to mediate the insulin-induced inhibition of glycogen synthase kinase-3 (GSK3) in L6 cultured myotubes; this inhibition is thought to underlie, at least in part, the insulin-induced dephosphorylation and activation of glycogen synthase [9] and protein synthesis initiation factor eIF2 [10] that contribute to the stimulation of glycogen and protein synthesis by insulin. PKB is likely to have other physiological substrates, however, and in transfection experiments it has been shown to activate p70 S6 kinase [5], to stimulate the translocation of the glucose transporter GLUT4 to the plasma membrane and enhance glucose uptake in 3T3-L1 adipocytes [11], and to mediate the survival of neurones [12] and fibroblasts [13] induced by insulin-like growth factor 1 (IGF1).

A critical question concerns the mechanism by which PI 3-kinase triggers the activation of PKB. Activation of PKB is accompanied by its phosphorylation [5,14], and we have recently shown that activation by insulin or IGF1 results from phosphorylation of PKB at two residues, Thr308 and Ser473 [15]. Moreover, the insulin-induced or IGF1-induced phosphorylation of both residues is abolished by wortmannin, an inhibitor of PI 3-kinase [15]. These findings suggest that the protein kinase(s) which phosphorylate PKB at Thr308 and Ser473 might themselves be activated by phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P_3), the product of the reaction catalyzed by PI 3-kinase. In this paper, we demonstrate that this is indeed the case, and report the purification and initial characterization of a 3-phosphoinositide-dependent protein kinase, PDK1, which activates PKB by phosphorylating it at Thr308.

Figure 1



SDS–polyacrylamide gel of purified GST–PKBα. Human 293 cells were transiently transfected with the pEBG2T DNA construct expressing GST–PKBα, serum starved for 16 h and, after cell lysis, GST–PKBα was purified by affinity chromatography on glutathione–Sepharose (see Materials and methods). The glutathione–Sepharose eluate (3 μg protein) was electrophoresed on a 10% SDS–polyacrylamide gel and stained with Coomassie blue. The position of the molecular mass markers glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) are indicated.

Results

Purification of GST–PKBα from 293 cells

PKBα was expressed in human embryonic kidney 293 cells as a fusion protein with glutathione S-transferase (GST) attached at its amino terminus, and purified on glutathione–Sepharose beads. The preparation showed a major protein band that stained with Coomassie blue, of apparent molecular mass 85 kDa, corresponding to GST–PKBα (Fig. 1). The purity estimated by densitometric analysis of the gels was more than 70% and 2 mg of purified GST–PKBα was obtained routinely in each preparation made from forty 10 cm diameter dishes of 293 cells. GST–PKBα from unstimulated 293 cells had a very low activity, but it was activated 20-fold and 45-fold after stimulation of 293 cells with insulin and IGF1 (data not shown), respectively, indicating that it is recognized by the upstream protein kinase(s) that activate PKBα *in vivo*. GST–PKBα from unstimulated 293 cells was therefore used as the substrate with which to identify upstream activators.

Identification of a PtdIns(3,4,5)P_3-dependent protein kinase that activates PKBα

We identified an activator of GST–PKBα in skeletal muscle cytosol that eluted from a QAE–Sephadex column at 0.25 M salt, and we purified it a further 30 000-fold from the QAE–Sephadex eluate (Table 1 and Fig. 2). The activator was completely dependent on PtdIns(3,4,5)P_3 for activity, and, because it phosphorylates PKBα (see below), it is hereafter termed PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK1).

PDK activity could not be detected in the crude cytosol, but assuming a recovery of 50% through the initial batch-wise chromatography on QAE–Sephadex, the overall purification was about 500 000-fold, and the entire preparation procedure could be completed within 36 hours. PDK activity eluted as a single peak from SP-Sepharose (Fig. 2a), but resolved into two peaks on heparin–Sepharose (Fig. 2b); the minor component (peak 1) eluted at 0.5 M NaCl and the major component (peak 2) at 0.7 M NaCl. All further experiments were carried out with the PDK activity of peak 2 further purified through Mono S (Fig. 2c), unless stated otherwise. After the final purification step, the active fraction, which was devoid of PKB activity, comprised three major protein bands, with apparent molecular masses of 85, 67 and 45 kDa (data not shown). Only the 67 kDa band became phosphorylated upon incubation with Mg–ATP and phosphorylation was greatly increased in the presence

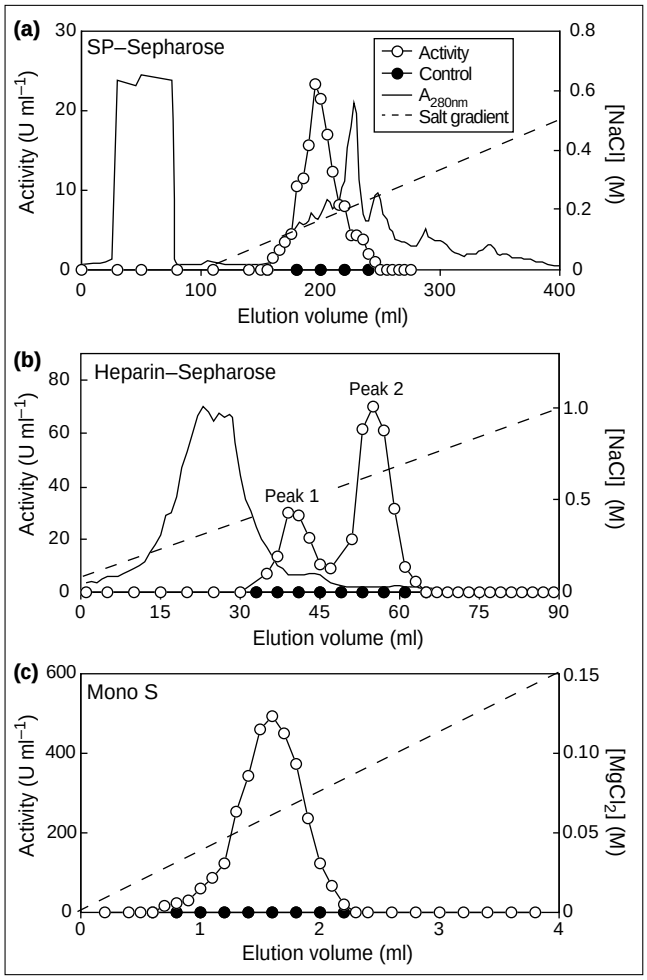
Table 1

Purification of PDK1 from rabbit skeletal muscle.

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
1. Extract	1 400	17 000	Not measurable	–	–	–
2. QAE–Sephadex	750	555	1 330	2.4	1	100
3. 4–15% PEG	55	164	1 190	7.3	3	89
4. SP–Sepharose	88	10	610	61	25	46
5. Heparin–Sepharose	11	0.01	290	29 000	12 000	22
6. Mono S	0.3	0.002	139	69 500	29 000	10

The amount of muscle used in this preparation was 500 g; the amount of protein at each step was estimated by the procedure of Bradford [28]. PEG is polyethyleneglycol.

Figure 2



Identification and purification of a PtdIns(3,4,5)P_3-dependent protein kinase, PDK1, which activates GST–PKBα. Following the PEG precipitation step, PDK activity was chromatographed sequentially on (a) SP–Sepharose and (b) heparin–Sepharose, and (c) peak 2 from the latter column was chromatographed on Mono S (see Materials and methods). PDK activity was assayed in the presence of either phospholipid vesicles comprising 100 μM phosphatidylcholine (PtdCho), 100 μM phosphatidylserine (PtdSer), 10 μM D-enantiomer of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 or vesicles comprising only 100 μM PtdCho and 100 μM PtdSer (control). The amount of protein eluted from the Mono S column was too low to see any absorbance at 280 nm.

of PtdIns(3,4,5)P_3 (data not shown). The amino-acid sequences of two tryptic peptides from the 67 kDa band were highly homologous to regions of the catalytic domains of other protein kinases (data not shown). The data indicate that PDK1 is a newly identified 67 kDa protein kinase, distinct from other protein kinases that have been reported to be activated by PtdIns(3,4,5)P_3, such as PKCε and PKCζ.

Phosphorylation and activation of GST–PKBα by PDK1

Purified PDK1 phosphorylated GST–PKBα in the presence of Mg–ATP and phospholipid vesicles containing phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer) and PtdIns(3,4,5)P_3, to a level approaching 0.7 moles of incorporated phosphate per mole of protein (Fig. 3a). Phosphorylation of GST–PKBα was paralleled by an increase in its activity of over 30-fold (Fig. 3a) to a specific activity of 80 U mg^{-1}. This is similar to the specific activity of PKBα that has been partially activated by a Thr308→Asp mutation [15,16]. Omitting either PtdIns(3,4,5)P_3, PDK1 or ATP from the reaction abolished activation and phosphorylation of GST–PKBα (Fig. 3b). Moreover, addition of 0.5% (by volume) Triton X-100 to the assays also prevented the activation and phosphorylation of GST–PKBα by PDK1 (Fig. 3b), as did incubating PDK1 for 2 minutes at 55°C. In the absence of PtdCho/PtdSer vesicles, PtdIns(3,4,5)P_3 was at least 15-fold less effective in activating PDK1 (Fig. 3b).

When PDK1 was incubated for 30 minutes at 30°C in the presence of phospholipid vesicles containing PtdCho, PtdSer, PtdIns(3,4,5)P_3 and Mg–ATP, and then for 2 minutes at 55°C to inactivate PDK1, the phospholipid vesicles were unable to activate or phosphorylate GST–PKBα unless more PDK1 was added (data not shown). These observations establish that PDK1 does not activate GST–PKBα indirectly by converting PtdIns(3,4,5)P_3 to another product that stimulates the autophosphorylation and autoactivation of GST–PKBα.

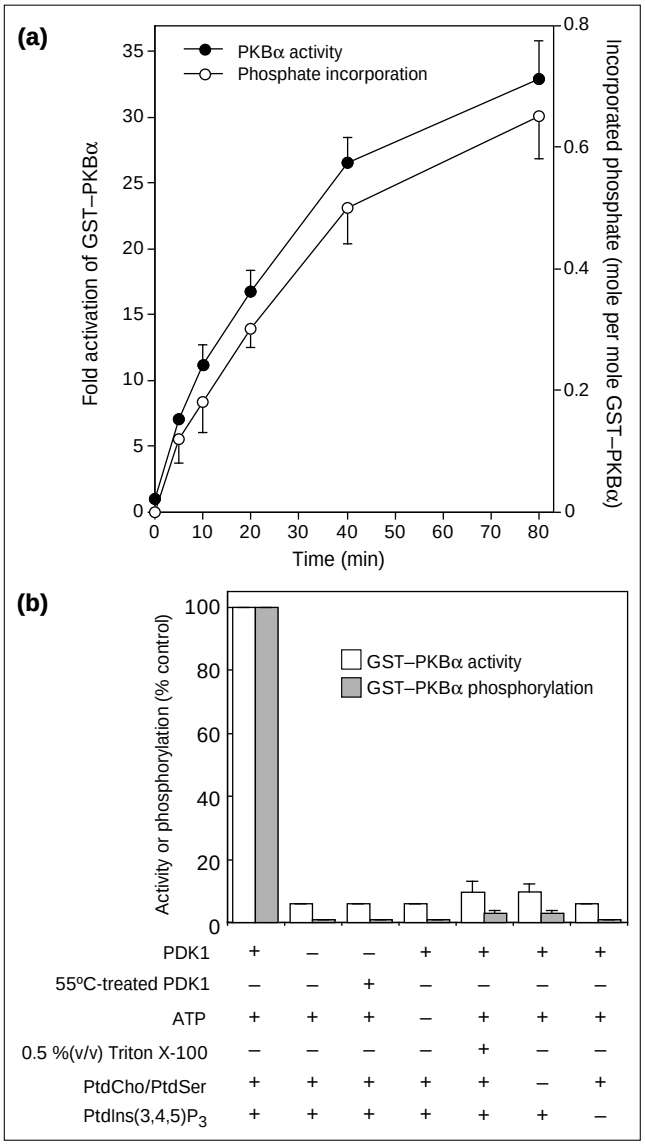
Dependence of PDK1 activity on PtdIns(3,4,5)P_3 concentration

We next investigated the effect of varying the PtdIns(3,4,5)P_3 concentration on the ability of PDK1 to activate (Fig. 4a) and phosphorylate (Fig. 4b,c) GST–PKBα. These experiments were carried out either by varying the concentration of PtdIns(3,4,5)P_3 whilst maintaining the concentration of both PtdCho and PtdSer at 100 μM, or by maintaining a 10-fold excess of PtdCho and PtdSer over PtdIns(3,4,5)P_3. Under both conditions, the concentration of PtdIns(3,4,5)P_3 required for half-maximal activation or phosphorylation was 1–2 μM, with a maximal effect at 10 μM.

PDK1 phosphorylates Thr308 of PKBα

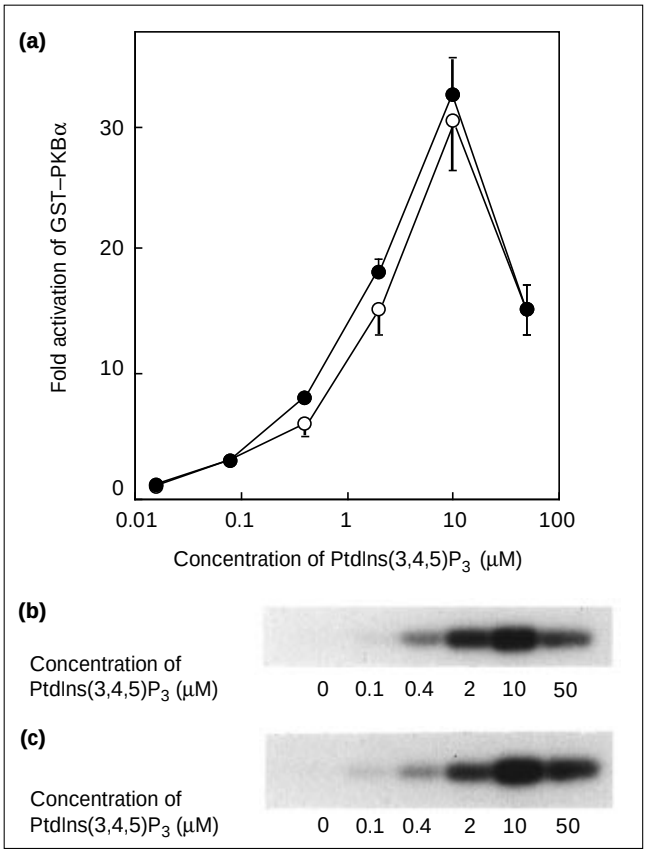
In order to identify the residue(s) in PKBα phosphorylated by PDK1, ^{32}P-labelled GST–PKBα which had been phosphorylated to 0.4–0.6 moles of phosphate per mole with PDK1, was digested with trypsin and chromatographed on a C_{18} column [15]. One major ^{32}P-labelled peptide was observed, which eluted at 26% acetonitrile (Fig. 5a). This peptide, which co-eluted with the ^{32}P-labelled tryptic phosphopeptide of PKBα containing Thr308 [15], was found to contain phosphate only on threonine (data not shown). When this peptide was subjected to solid-phase sequencing, ^{32}P- label was released after the first cycle of Edman degradation (Fig. 5b), corresponding to Thr308 [15]. Importantly, no ^{32}P-labelled peptide eluted at the position corresponding to the tryptic phosphopeptide

Figure 3



PDK1 phosphorylates and activates GST–PKBα. (**a**) GST–PKBα (0.5 μM) was incubated with PDK1 (12 U ml^{-1}), 10 mM $Mg(Ac)_2$, 100 μM γ-[^{32}P]ATP, and vesicles comprising 100 μM PtdCho, 100 μM PtdSer, 10 μM D enantiomer of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 in buffer B (see Materials and methods). At various times, aliquots were removed and assayed either for PKBα activity or for incorporation of phosphate into GST–PKBα. The activity of GST–PKBα is presented relative to its activity in control experiments in which PDK1 was omitted from the reaction mixture. Phosphorylation was assessed by counting the radioactive phosphate (^{32}P) incorporated into the GST–PKBα band after SDS–PAGE. (**b**) The same assays were carried out as in (a) except that the effects of omitting either PDK1, ATP, PtdSer/PtdCho, or PtdIns(3,4,5)P_3, heating PDK1 at 55°C for 2 min, or adding 0.5% (by volume) Triton X-100 on the PDK1 assay at the 60 min time point were studied. GST–PKBα activity and GST–PKBα phosphorylation are shown relative to control assays in which PDK1, ATP, PtdCho/PtdSer and PtdIns(3,4,5)P_3 were present. The results are presented as the mean ± SEM of six determinations (two independent experiments).

Figure 4



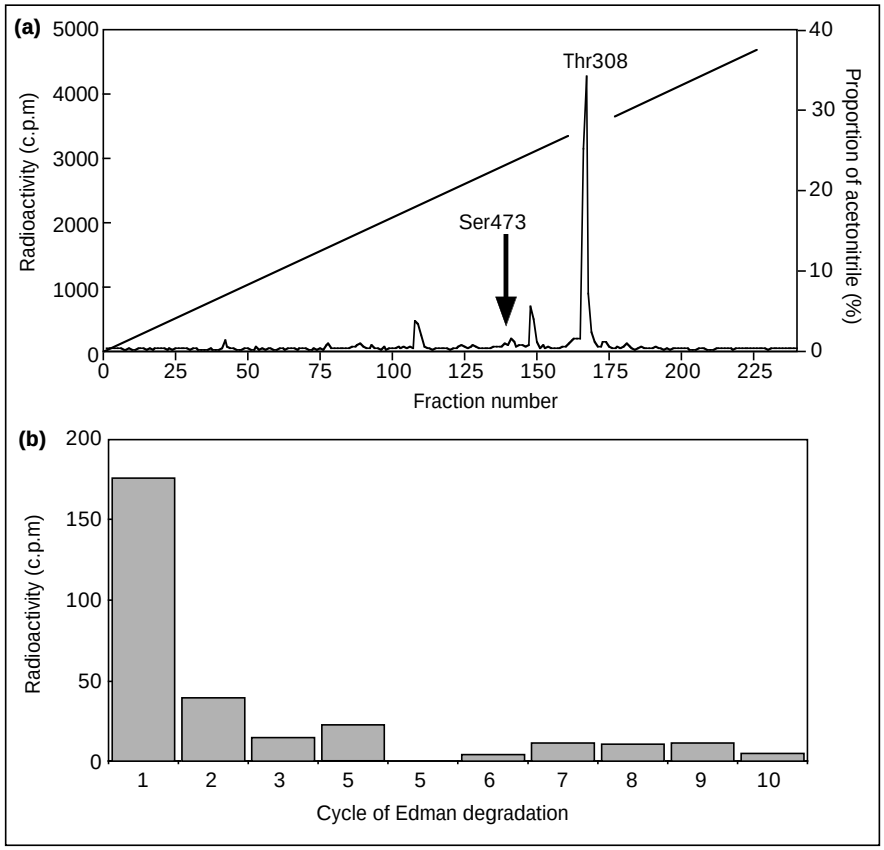
Dependence of PDK1 activity on PtdIns(3,4,5)P_3 concentration. The extent of (**a**) activation and (**b**,**c**) phosphorylation of 0.5 μM GST–PKBα was assessed (as described in Materials and methods) after 30 min incubation with PDK1 (12 U ml^{-1}), 10 mM MgAc, 100 μM γ-[^{32}P]ATP and the indicated concentrations of D enantiomer of 1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 either in the presence of a constant concentration of PtdCho and PtdSer (100 μM of each; b and open circles in a) or in the presence of a 10-fold molar excess of both PtdSer and PtdCho over PtdIns(3,4,5)P_3 (c and closed circles in a). The results are presented as the mean ± SEM of two experiments, each carried out in triplicate.

containing Ser473, which elutes at 24% acetonitrile (Fig. 5a; [15]). These data establish that, *in vitro*, PDK1 phosphorylates PKBα at Thr308 only.

Lipid specificity of PDK1

We next studied the ability of a panel of PtdIns derivatives, presented in a vesicle background containing PtdCho/PtdSer, to activate PDK1. The predominant form of PtdIns(3,4,5)P_3 that occurs naturally is likely to be *sn*-1-stearoyl, 2-arachidonyl D-PtdIns(3,4,5)P_3 [17] (on the basis of the structure and fatty acid composition of natural PtdIns). Synthetic *sn*-1-stearoyl, 2-arachidonyl D-PtdIns(3,4,5,)P_3 — lipid 1 in Figure 6 — proved highly effective, activating PDK1 activity 13-fold. By contrast, the L enantiomer of this lipid induced only a 1.7-fold

Figure 5



PDK1 phosphorylates PKBα at Thr308. (a) GST–PKBα was phosphorylated by incubation for 60 min with PDK1 (12 U ml^{-1}) and phospholipid vesicles comprising PtdCho, PtdSer, D enantiomer of 1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3, and Mg–γ-[^{32}P]ATP (see legend to Fig. 3), and then alkylated and digested with trypsin. The digest was applied to a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, California) equilibrated in 0.1% (by volume) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml min^{-1} and fractions of 0.4 ml were collected. About 35% of the radioactivity applied to the column was recovered from the major ^{32}P-containing peptide which eluted at 26% acetonitrile (the remainder of the radioactivity eluted as numerous minor peaks). The elution positions of PKBα tryptic phosphopeptides which contain Thr308 and Ser473 are marked [15]. (b) A portion of the major ^{32}P-containing peptide (500 c.p.m.) from (a) was covalently coupled to a Sequelon arylamine membrane and analyzed on an Applied Biosystems 470A sequencer using the modified programme described previously [29]. The released radioactivity was measured after each cycle of Edman degradation.

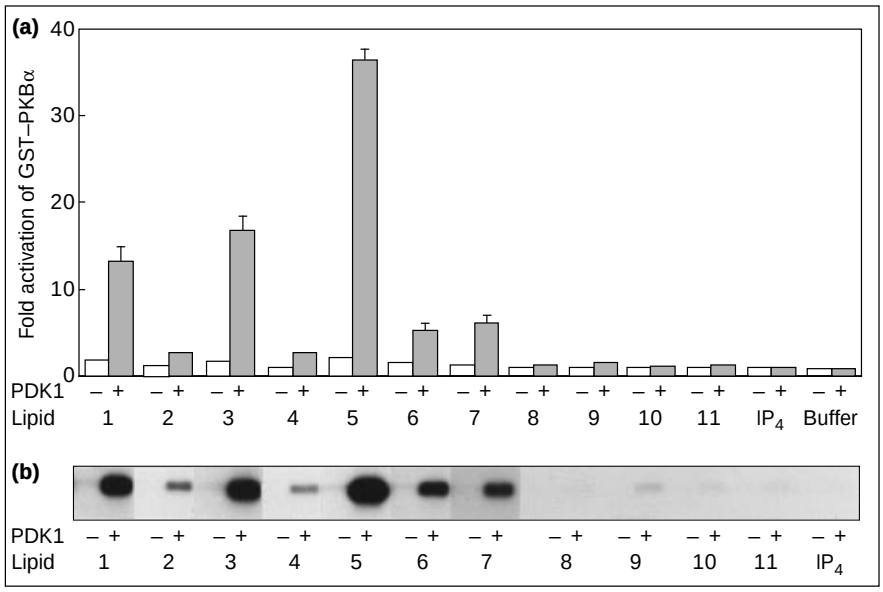
increase in PDK1 activity, which might be accounted for by trace contamination with the D enantiomer. Whereas the enantiomeric configuration of the head group was of critical importance for activating PDK1, that of the glycerol moiety was not. Thus, *sn*-2-arachidonyl, 3-stearoyl D- and L-PtdIns(3,4,5)P_3 gave signals which were indistinguishable from the *sn*-1,2-derivatives (16-fold and 2.3-fold, respectively). The importance of unsaturated fatty acids in the diacylglycerol moiety is strongly indicated by the fact that racemic *sn*-1,2-dilinoleoyl PtdIns(3,4,5)P_3 was the most effective lipid tested (linoleic acid is C18:2), causing a 36-fold increase in GST–PKBα activity, whereas *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3 induced only a 5.5-fold activation (palmitic acid is C16:0). In each of these experiments, the phosphorylation of GST–PKBα (Fig. 6b) correlated with the extent of activation (Fig. 6a).

Interestingly, *sn*-1,2-dipalmitoyl PtdIns(3,4)P_2 (lipid 7 in Fig. 6) and *sn*-1,2-dipalmitoyl PtdIns(3,4,5)P_3 (lipid 6 in Fig. 6) activated PDK1 to the same extent , both inositol phospholipids increasing GST–PKBα activity about 6-fold. However, PtdIns(3,5)P_2 (lipid 8 in Fig. 6), PtdIns(4,5)P_2 (lipid 9), PtdIns 4P (lipid 10), PtdIns 3P (lipid 11) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) did not activate PDK1 or induce the phosphorylation of GST–PKBα. In the absence of PDK1, none of the PtdIns derivatives tested induced any activation or phosphorylation of GST–PKBα (Fig. 6).

Discussion

The identification and purification of PDK1 was greatly facilitated by the development of a specific peptide substrate (RPRAATF, in single-letter amino-acid code) for PKBα [16]. Other substrates used to assay PKB, such as histone H2B, myelin basic protein and Crosstide, are phosphorylated by many protein kinases in cell extracts and therefore obscured the detection of PDK1. The use of soluble GST–PKBα as a substrate was also important because PDK1 did not phosphorylate immunoprecipitated haemagglutinin-tagged PKB effectively. We found that PDK1 phosphorylated PKBα at Thr308 and enhanced its activity over 30-fold. As the phosphorylation of Thr308 of PKBα induced by insulin or IGF1 *in vivo* is prevented by inhibitors of PtdIns 3-kinase, and as PDK1 has an absolute requirement for PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, PDK1 is likely to be the enzyme which phosphorylates PKBα at Thr308 *in vivo*. PDK1 activity is unaffected by wortmannin at concentrations of up to 2 μM (data not shown), indicating that PDK1 is not a PI 3-kinase family member.

Figure 6



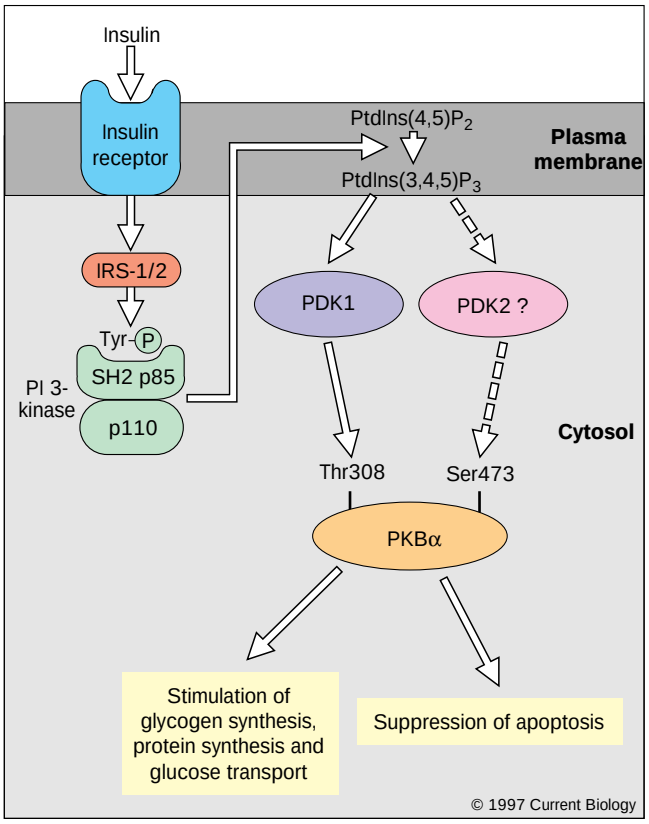
PDK1 is activated specifically and stereospecifically by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 lipids. GST–PKBα was incubated for 30 min at 30°C with Mg–γ-[^{32}P]ATP and phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer, and various PtdIns lipids (numbered 1–11; see below) or Ins(1,3,4,5)P_4, each at a final concentration of 10 μM, in the presence or absence of PDK1. Reactions were terminated by the addition of 1% (by volume) Triton X-100 to the assay. **(a)** The activity of GST–PKBα was determined as described in Materials and methods. The results are presented as the mean fold activation of GST–PKBα ± SEM for six to eight determinations (three independent experiments). **(b)** The phosphorylation of GST–PKBα was assessed by autoradiography of the Coomassie-blue-stained band corresponding to GST–PKBα (see Fig. 1 legend). Lipids 1 and 2 are the D and L enantiomers of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3, respectively, and lipids 3 and 4 are the D and L enantiomers of *sn*-2-arachidonyl, 3-stearoyl PtdIns(3,4,5)P_3, respectively. Lipid 5 is racemic *sn*-1,2-dilinoleoyl PtdIns(3,4,5)P_3. The remaining lipids are all D enantiomers; lipid 6 is *sn*-1,2-dipalmitoyl PtdIns(3,4,5)P_3; lipid 7 is *sn*-1,2-dipalmitoyl PtdIns(3,4)P_2; lipid 8 is *sn*-1,2-dipalmitoyl PtdIns(3,5)P_2. Lipid 9 is PtdIns(4,5)P_2 and lipid 10 is PtdIns 4P; both are purified lipids derived from Folch fraction type-I brain extract. Lipid 11 is *sn*-1,2-dipalmitoyl PtdIns-3P. IP_4 is Ins(1,3,4,5)P_4.

The full activation of PKBα *in vitro* requires the phosphorylation of Ser473 as well as Thr308 [15]. Thus, mutation of either Thr308 or Ser473 to aspartic acid stimulates PKBα activity about 5-fold but, if both residues are mutated to aspartic acid, activity is increased 18-fold. Similarly, phosphorylation of Ser473 stimulates PKBα activity 7-fold, but if phosphorylation of Ser473 is combined with mutation of Thr308 to aspartic acid, then activity is increased 25-fold [15]. PKBα that has been partially activated either by phosphorylation of Thr308 or by mutation of this residue to aspartic acid does not become phosphorylated at Ser473 *in vitro* upon incubation with Mg–ATP in the presence of PtdIns(3,4,5)P_3 ([15] and Fig. 5), indicating that Ser473 is unlikely to be autophosphorylated by PKBα itself. Ser473 can be phosphorylated *in vitro* by mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK2), but this enzyme cannot mediate the insulin-induced or IGF1-induced phosphorylation of PKBα at Ser473 for reasons we have discussed previously [15]. As the insulin/IGF1-induced phosphorylation of Ser473 is prevented by inhibitors of PI 3-kinase, this residue may be phosphorylated by a distinct 3-phosphoinositide-dependent protein kinase (perhaps PDK2; see Fig. 7). The Ser473 kinase does not appear to be the PtdIns(3,4,5)P_3-dependent peak 1 activity seen after the heparin–Sepharose PDK1 purification step (Fig. 2b), however, because this enzyme also phosphorylates PKB at Thr308 (data not shown). The peak 1 activity may be a proteolytic fragment from peak 2 (or *vice versa*), or maybe another isoform of PDK1. It is also possible that PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 activates the Ser473 kinase indirectly. For example, the phosphorylation of Ser473 may be dependent on the binding of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 to the pleckstrin homology (PH) domain of PKB [18,19] (but see below). Alternatively, the Ser473 kinase may be activated by PDK1. The mechanism we propose for the activation of PKBα following insulin stimulation is shown schematically in Figure 7.

The activation of PDK1 by PtdIns(3,4,5)P_3 is extremely specific, because only D enantiomers of PtdIns(3,4,5)P_3 are effective and many other PtdIns phospholipids are not. Although the enantiomeric configuration of the glycerol moiety is not important, the presence of one or more unsaturated fatty acids greatly influences the extent of activation of PDK1 by PtdIns(3,4,5)P_3 analogues (Fig. 6). Since unsaturated fatty acids discourage tight packing of adjacent phospholipid molecules, it is possible that this arrangement allows for more efficient interaction between membrane-inserted PtdIns(3,4,5)P_3 and its effectors, perhaps explaining for the first time the biological significance of the unusual fatty acid composition of inositol phospholipids.

PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 have no effect on the activity of PKBα under conditions where these inositol phospholipids activate PDK1 strongly (Fig. 6), consistent with our previous findings [18]. This observation, together

Figure 7



Proposed mechanism by which insulin induces the activation of PKBα. Activation of the insulin receptor by insulin causes the receptor to phosphorylate itself at several tyrosine residues. This leads to docking of insulin receptor substrate-1 (IRS-1) and IRS-2 and their phosphorylation at multiple tyrosine residues by the insulin receptor. Several phosphorylated tyrosine residues on IRS-1 and IRS-2 then interact with SH2 domains on the p85 subunit of PI 3-kinase, leading to the recruitment of the p110 catalytic subunit of PI 3-kinase to cell membranes, and its activation [30]. PI 3-kinase then phosphorylates PtdIns(4,5)P_2 at the D-3 position of the inositol ring, resulting in the formation of the second messenger PtdIns(3,4,5)P_3, levels of which (in a typical cell) rise from 0.1% to 1–2% of the PtdIns(4,5)P_2 content in the plasma membrane [30]. PtdIns(3,4,5)P_3 interacts with and activates PDK1, which partially activates PKBα by phosphorylating PKBα at Thr308. Maximal activation of PKBα also requires the phosphorylation of Ser473 by an unknown kinase. The insulin-induced phosphorylation of Ser473, like the phosphorylation of Thr308, is prevented by inhibitors of PI 3-kinase [15], suggesting that Ser473 may be phosphorylated by a distinct 3-phosphoinositide-dependent protein kinase (PDK2). The Ser473 kinase may, however, be activated indirectly by 3-phosphoinositides by mechanisms discussed in the text. For this reason, the pathway from PtdIns(3,4,5)P_3 to the phosphorylation of Ser473 by the putative PDK2 is shown by broken lines. In skeletal muscle, the activation of PKBα may increase the rate of glucose uptake [11] and glycogen synthesis [9] and stimulate protein translation [10]. In neurons and other cells, PKBα activation may provide a survival signal by suppressing apoptosis [12,13].

with the finding that the activation of PKBα by insulin or IGF1 results from its phosphorylation at Thr308 and Ser473 [15], appears to exclude direct activation of PKBα by 3-phosphoinositides as a mechanism *in vivo*. Our results disagree with recent reports which have claimed that PKBα is activated directly by PtdIns(3,4)P_2 [19–21]. Contamination of PKBα preparations with PDK1 activity may explain this discrepancy; it is worth recalling that the activation of phosphorylase kinase by cyclic AMP [22] was later shown to result from contamination with a separate cyclic AMP-dependent protein kinase [23]. It has also been reported that PKBα is inhibited by PtdIns(3,4,5)P_3 [19,21], but none of the four PtdIns(3,4,5)P_3 derivatives we tested inhibited the basal PKBα activity at all, while all four were capable of activating PDK1. It is possible that the synthetic PtdIns(3,4,5)P_3 used in previous studies [19,21] contains impurities that inhibit PKBα and/or PDK1.

Although PKB is not activated directly by PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, it does bind these inositol phospholipids with micromolar affinity [18,19], by the amino-terminal PH domain [19]. In contrast, PKB binds PtdIns(4,5)P_2 with a 10-fold lower affinity and does not bind to other inositol phospholipids tested [18,19]. These findings raise several interesting possibilities. Firstly, the interaction of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 with PKB may alter its conformation in such a way as to facilitate phosphorylation by PDK1 and the Ser473 kinase. Secondly, the formation of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 in the plasma membrane may recruit PKB to this membrane, also facilitating its activation by PDK1 and the Ser473 kinase. Neither of these mechanisms appears to be essential for the activation of PKB *in vivo*, however, because a mutant lacking the PH domain can still be activated at least as well as wild-type PKB in response to insulin [6,24]. Alternatively, recruitment of PKB to the plasma membrane could facilitate the phosphorylation of membrane-bound PKB substrates. It should be noted, however, that we purified PDK1 from the cytosol of skeletal muscle. It will clearly be important to examine whether PDK1 localizes to the plasma membrane when cells are stimulated with agonists which trigger a rise in the concentration of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.

Materials and methods

Materials

PtdSer (pig brain) was purchased from Doosan Serdary Research Laboratories (New Jersey, USA) and *sn*-1-stearoyl, 2-arachidonyl PtdCho from Sigma (Poole, UK). PtdIns 4P and PtdIns(4,5)P_2 were purified as described previously from Folch fraction type-1 extract of bovine brain (Sigma) [25]. Synthetic *sn*-1,2-dipalmitoyl analogues of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns 3P were made as described previously [18]. Synthetic D and L enantiomers of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3, *sn*-2-arachidonyl, 3-stearoyl PtdIns(3,4,5)P_3 and the racemic mixture of *sn*-1-linoleoyl-2-linoleoyl PtdIns(3,4,5)P_3 were synthesized from inositol (P.R.J.G. and C.B.R., unpublished methods). All phospholipids were 97–98% pure. Synthetic phosphatidylinositol bisphosphates were stored at –20°C as solutions in dimethyl sulphoxide (DMSO), and phosphatidylinositol trisphosphates were stored in either DMSO or water. All other phospholipids were stored in chloroform–methanol solvents. The peptide used to assay PKBα, (RPRAATF) [16] and TTYADFIASGRTGRRNAIHD (the specific

peptide inhibitor of cyclic-AMP-dependent protein kinase, termed PKI) were synthesized by F. Barry Caudwell (Medical Research Council Protein Phosphorylation Unit, Dundee) on an Applied Biosystems 431A peptide synthesizer, and their concentrations were determined by quantitative amino-acid analysis. Glutathione–Sepharose was purchased from Pharmacia (Milton Keynes, UK) and alkylated trypsin from Promega (Southampton, UK).

Buffer solutions

Buffer A contained 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (by volume) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg ml^{-1} leupeptin and 0.1% (by volume) 2-mercaptoethanol. Buffer B contained 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.03% (by volume) Brij-35, 0.27 M sucrose and 0.1% (by volume) 2-mercaptoethanol. Buffer C contained 50 mM Tris/HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM PMSF and 1 mM benzamidine.

Expression of GST–PKBα in 293 cells

The DNA sequence encoding PKBα was subcloned into the eukaryotic expression vector PEBG2T which has been used to overexpress GST-fusion proteins in eukaryotic cells under an EF1α promoter [26]. A polymerase chain reaction (PCR) was set up to generate a full-length cDNA encoding the PKBα gene with a *Bam*H1site at the 5′ end of the cDNA that was in frame with the GST sequence of the PEBG2T vector and the ATG initiation codon of PKBα, and a *Kpn*I site at the 3′ end, using the human PCMV5–HA–PKBα plasmid [15]. The DNA sequence of the *Bam*H1–*Kpn*I cDNA fragment was checked and then subcloned into the unique *Bam*H1–*Kpn*I restriction sites on the pEBG2T expression vector. In order to prepare GST–PKBα, forty 10 cm diameter dishes of human embryonic kidney 293 cells were cultured and each dish transfected with 20 μg GST–PKBα expression construct using the modified calcium phosphate method described previously for overexpression of haemagglutinin-tagged PKBα in 293 cells [15]; 24 h after transfection, the cells were serum starved for 16 h and each dish of cells was lysed in 1 ml ice-cold Buffer A. The 40 lysates were pooled, centrifuged at 4°C for 10 min at 13 000 *g* and the supernatant incubated for 60 min on a rotating platform with 800 μl glutathione–Sepharose previously equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3000 *g*, the beads washed three times with 10 ml Buffer A containing 0.5 M NaCl, and then a further 10 times with 10 ml Buffer B to ensure complete removal of all the Triton X-100 which interferes with the activation of GST–PKBα by PDK1; Figure 3b. GST–PKBα was eluted from the resin at ambient temperature with three 1 ml portions of Buffer B containing 20 mM glutathione pH 8.0. The combined eluates (0.65 mg protein per ml) were divided into aliquots, snap frozen in liquid nitrogen, and stored at –80°C.

Preparation of phospholipid vesicles

Phospholipid vesicles comprising 1 mM PtdCho, 1 mM PtdSer and 0.1 mM PtdIns lipids were prepared, dried to a film under vacuum and resuspended with vortexing in 10 mM Hepes, pH 7.3. The cloudy solution of multilamellar and large unilamellar vesicles was bath-sonicated for 20 min, after which a clearer suspension of smaller unilamellar vesicles was obtained. Solutions were stored at 4°C at concentrations 10-fold higher than those required in the assay, and used within 2–4 days.

Purification of PDK1 from rabbit skeletal muscle

On day 1, a female New Zealand White rabbit was killed with a lethal dose of sodium pentobarbitone. Skeletal muscle from the hind limbs and back (500 g) was excised rapidly and placed on ice. All subsequent steps were carried out at 0–4°C. The muscle was minced, homogenized in 2.5 volumes of 25 mM Tris/HCl pH 7.5, 4 mM EDTA, 2 mM EGTA, 50 mM NaF, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM PMSF and 1 mM benzamidine and centrifuged for 30 min at 4200 *g*. The supernatant was filtered through glass wool and poured through a Buchner funnel containing 10 g of QAE (quaternany amino ethyl)–Sephadex equilibrated in Buffer C. The column was washed with 1 l Buffer C containing 50 mM NaCl, and washed with 700 ml Buffer C containing 0.2 M NaCl to elute PDK1. A 50% (by mass) solution of polyethylene glycol (PEG) was added to the eluate to bring the final concentration of PEG to 4%. After stirring on ice for 60 min, the suspension was centrifuged for 15 min at 10 000 *g*. The supernatant was decanted and made up to 15% PEG by further addition of 50% (by mass) PEG. After stirring for a further 60 min on ice, the suspension was again centrifuged for 15 min at 10 000 *g*. The supernatant was discarded and the precipitate dissolved in 50 ml ice-cold Buffer B, filtered through a 0.25 μm filter and then chromatographed on an SP–Sepharose column (11 × 1.6 cm) equilibrated in Buffer B. The column was developed with a 300 ml linear gradient of NaCl to 0.5 M at a flow rate of 3 ml min^{-1} and 6 ml fractions were collected (Fig. 2a). The active fractions were pooled and applied directly onto a 5 ml heparin–Sepharose column (HiTrap), equilibrated in Buffer B containing 0.1 M NaCl. The column was developed with a 90 ml linear salt gradient to 0.9 M NaCl at a flow rate of 2 ml min^{-1} and fractions of 1 ml were collected. PDK activity eluted as 2 peaks; peak 1, comprising ~30% of the activity, eluted at 0.5 M NaCl, whereas peak 2, comprising ~70% of the activity, eluted at 0.7 M NaCl (Fig. 2b). On day 2, the active fractions of peak 2 were pooled, concentrated to 0.2 ml by ultrafiltration, diluted to 2.0 ml in Buffer B, and loaded onto a Mono S column (5 cm × 1.6 mm) equilibrated in Buffer B. The column was developed with a 4.0 ml linear gradient of salt to 0.15 M $MgCl_2$ at a flow rate of 0.2 ml min^{-1}, and 0.1 ml fractions were collected. Fractions containing PDK activity (PDK1) were aliquoted, snap-frozen in liquid nitrogen, and stored at –80°C. No significant loss of activity occurred upon thawing.

Assay of PDK1

The assay was carried out in two stages; in the first stage, GST–PKBα was incubated with PDK1 in the presence of Mg–ATP and phospholipid vesicles to permit activation of GST–PKBα. In the second stage, the solution was made up to 0.5 % (by volume) Triton X-100 (which completely inhibits phosphorylation and activation of GST–PKBα by PDK1 without affecting GST–PKBα activity; see Fig. 3b), together with Mg–γ-[^{32}P]ATP, and the specific PKBα substrate peptide RPRTAAF [16].

In Stage 1, an 18 μl reaction mixture was set up containing 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM EGTA, 2.5 μM PKI, 1 μM microcystin-LR, 10 mM $Mg(Ac)_2$, 100 μM unlabelled ATP, 0.6 μM GST–PKBα, 100 μM PtdSer, 100 μM PtdCho in the presence or absence of 10 μM PtdIns(3,4,5)P_3. The assay was initiated by the addition of 2 μl PDK1 and, after incubation for 30 min at 30°C, stage 2 of the assay was initiated by the addition of 30 μl of a mixture containing 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM EGTA, 2.5 μM PKI, 1 μM microcystin-LR, 10 mM $Mg(Ac)_2$, 100 μM γ-[^{32}P]ATP (200–400 c.p.m. $pmol^{-1}$), 100 μM of the peptide RPRTAAF and 1.25% (by volume) Triton X-100. After 10 min at 30°C, the reactions were terminated by spotting the reaction mixture onto p81 phosphocellulose paper. The papers were washed in phosphoric acid and analysed as described previously [27]. A control reaction in which GST–PKBα was omitted was taken as the blank and was always less than 5% of the activity measured in the presence of GST–PKBα. The basal GST–PKBα activity is the activity measured in the absence of PDK1. One unit (U) of PDK1 activity was defined as the amount required to increase the basal activity of GST–PKBα by 1 unit of activity; 1 unit of GST–PKBα activity was the amount of enzyme required to catalyze the phosphorylation of 1 nmol of the peptide RPRTAAF in 1 min. The assays were linear with time, up to a final concentration of 2 U ml^{-1} PDK1 activity in the assay.

Phosphorylation of GST–PKBα by PDK1

The incubations were identical to those in stage 1 described above, except that γ-[^{32}P]ATP (200–400 c.p.m. $pmol^{-1}$) was used instead of unlabelled ATP. The reactions were terminated by making the solution 1% (by mass) SDS. The samples were run on a 7.5% SDS–polyacrylamide gel and, after staining with Coomassie blue, the gels were

autoradiographed. The Coomassie-blue-staining bands corresponding to GST–PKBα were excised and the radioactivity associated with each band was quantified.

Mapping the site on GST–PKBα phosphorylated by PDK1

GST–PKBα was phosphorylated in the presence of the D enantiomer of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 as described above, except that the scale of the reaction was increased 10-fold and the specific activity of the γ-[^{32}P]ATP was increased to 1500 c.p.m. pmol^{-1}. The reaction was stopped by the addition of SDS and 2-mercaptoethanol to final concentrations of 1% (by mass) and 1% (by volume), respectively, and heated for 5 min at 95°C. After cooling to ambient temperature, 4-vinylpyridine was added to a concentration of 2.5% (by volume) and the sample left on a shaking platform for 1 h at 30°C to alkylate cysteine residues. The sample was then electrophoresed on a 7.5% SDS–polyacrylamide gel, and the ^{32}P-labelled GST–PKBα eluted from the gel and digested with trypsin as described previously [15].

Acknowledgements

We thank M. Andjelkovic and B.A. Hemmings for providing the PKBα gene, A. Waskiewicz for the pEBG2T expression plasmid and F. Barry Caudwell for peptide synthesis and solid-phase amino-acid sequencing. This work was supported by the Medical Research Council (P.C., D.R.A. and C.P.D.), The Royal Society (P.C.), the British Diabetic Association (P.C.), Biotechnology and Biological Sciences Research Council (S.R.J. and C.P.D.), and the Wellcome Trust (P.R.J.G. and C.B.R.).

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