3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase

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Background: The activation of protein kinase B (PKB, also known as c-Akt) is stimulated by insulin or growth factors and results from its phosphorylation at Thr308 and Ser473. We recently identified a protein kinase, termed PDK1, that phosphorylates PKB at Thr308 only in the presence of lipid vesicles containing phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) or phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2).

Results: We have cloned and sequenced human PDK1. The 556-residue monomeric enzyme comprises a catalytic domain that is most similar to the PKA, PKB and PKC subfamily of protein kinases and a carboxy-terminal pleckstrin homology (PH) domain. The PDK1 gene is located on human chromosome 16p13.3 and is expressed ubiquitously in human tissues. Human PDK1 is homologous to the *Drosophila* protein kinase DSTPK61, which has been implicated in the regulation of sex differentiation, oogenesis and spermatogenesis. Expressed PDK1 and DSTPK61 phosphorylated Thr308 of PKBα only in the presence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. Overexpression of PDK1 in 293 cells activated PKBα and potentiated the IGF1-induced phosphorylation of PKBα at Thr308. Experiments in which the PH domains of either PDK1 or PKBα were deleted indicated that the binding of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 to PKBα is required for phosphorylation and activation by PDK1. IGF1 stimulation of 293 cells did not affect the activity or phosphorylation of PDK1.

Conclusions: PDK1 is likely to mediate the activation of PKB by insulin or growth factors. DSTPK61 is a *Drosophila* homologue of PDK1. The effect of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 in the activation of PKBα is at least partly substrate directed.

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Background

Protein kinase B (PKB) [1] or Rac protein kinase [2] is the cellular homologue of a viral oncogene product v-Akt [3] and has therefore also been termed c-Akt. The current interest in PKB stems, firstly, from the observation that it is activated rapidly in response to insulin and growth factors and that the activation is prevented by inhibitors of phosphoinositide (PtdIns) 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]. More recently, it has been found that PKB provides a survival signal that protects cells from apoptosis induced by ultraviolet radiation, by the withdrawal of the survival factor insulin-like growth factor-1 (IGF1) from neuronal cells, by the withdrawal of serum factors while c-*myc* is expressed artificially, and by detachment of cells from the extracellular matrix [9–12]. The activation of PKB by gene amplification (and other mechanisms) may, therefore, contribute to the generation of malignancies that are able to flourish in the absence of extracellular survival signals [13].

Only two of the physiological substrates for PKB have so far been identified. PKB appears to mediate the insulin-induced inhibition of glycogen synthase kinase-3 (GSK3); this is thought to underlie the insulin-induced dephosphorylation and activation of glycogen synthase [14] and protein synthesis initiation factor eIF-2B [15] that contributes to the stimulation of glycogen and protein synthesis by insulin [16]. PKB also appears to mediate the insulin-induced activation of the cardiac isoform of 6-phosphofructo-2-kinase, which is likely to underlie the stimulation of cardiac glycolysis by this hormone [17,18]. In transfection-based experiments, PKB also mimics two further actions of insulin, namely the activation of p70 S6 kinase [5]

and the enhancement of glucose uptake that results from the translocation of GLUT4 from an intracellular compartment to the plasma membrane [19].

The activation of PKB induced by insulin or IGF1 results from its phosphorylation at Thr308 and Ser473 and the phosphorylation of both of these residues *in vivo* is abolished by wortmannin, an inhibitor of PtdIns 3-kinase [20]. These findings suggested that the protein kinases that phosphorylate PKB at Thr308 and Ser473 would be effective in activating PKB only in the presence of phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P_3), the product of the PtdIns 3-kinase reaction. This proved to be the case when we reported recently the purification from skeletal muscle and initial characterisation of a protein kinase that phosphorylates PKB at Thr308 [21]. This enzyme activates PKBα only in the presence of either PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 and has, therefore, been termed 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 activity itself is unaffected by wortmannin, indicating that it is not a member of the PtdIns 3-kinase family [21].

In order to elucidate the role of PDK1 *in vivo* it was clearly essential to first determine the structure of the enzyme and the mechanism by which PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 allows PDK1 to phosphorylate PKB. In this paper, we report the cloning, sequencing and expression of human PDK1 and demonstrate that the expressed enzyme activates PKBα *in vitro* in an identical manner to PDK1 purified from muscle. PDK1 also activates PKBα in cotransfection experiments and potentiates the phosphorylation of Thr308 induced by IGF1. We demonstrate that the binding of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 to the pleckstrin homology (PH) domain of PKBα is necessary for PDK1 to phosphorylate and activate PKBα. Interestingly, PDK1 was found to be structurally and functionally homologous to the *Drosophila* protein kinase DSTPK61, which has been implicated in the regulation of sexual development in *Drosophila* (C.M., M. Todman and M.B., unpublished observations).

Results

Characterisation of PDK1 purified from rabbit skeletal muscle

PDK1 was purified 500,000-fold from rabbit skeletal muscle and about 2 μg of protein were isolated from 500 g of tissue [21]. SDS–polyacrylamide gel electrophoresis of the preparation revealed three proteins, two with molecular masses of 85 kDa and 45 kDa and a diffuse band of 67–69 kDa. Each band was excised from the gel, digested with trypsin and the resulting peptides chromatographed on a C_{18} column and sequenced. This analysis revealed that the 85 kDa and 45 kDa proteins were 6-phosphofructose 1-kinase and the α-subunit of casein kinase II, respectively, and that the 67–69 kDa band was likely to encode a previously unknown protein kinase. Since purified casein kinase II did not activate or phosphorylate a fusion protein of PKBα with glutathione S-transferase (GST–PKBα; data not shown), the 67–69 kDa protein was considered likely to be PDK1. This was consistent with the observation that PDK1 activity eluted from a gel filtration column with an apparent mass of ~70 kDa (data not shown). Evidence to be described below established this was indeed the case, and for this reason the protein is hereafter termed PDK1.

The human expressed sequence tag database dbEST was interrogated using nine peptide sequences from the 67–69 kDa band, which identified 14 overlapping sequences (Table 1) that together encode a novel protein kinase. A PCR fragment corresponding to part of this protein was generated from a human MCF7 cell line cDNA library, and this was used to isolate a full-length clone from the same library (see Materials and methods). The nucleotide and deduced amino acid sequence of PDK1 is shown in Figure 1. It contains all of the nine peptide sequences that we obtained from the 67–69 kDa protein from rabbit skeletal muscle without any amino acid changes, indicating that the protein sequence is highly conserved between mammals. The sequence encodes a 556-residue protein, with a predicted molecular mass of 63.1 kDa. We expressed the PDK1 protein with a Myc epitope tag in 293 cells (see below), the tagged protein migrating as a 68–70 kDa band in SDS–polyacrylamide gel electrophoresis. PDK1 antibodies that we have raised recognise a 69 kDa band in 293 cell lysates (data not shown). For this reason, and because the initiating methionine lies in a good Kozak consensus sequence, the sequence shown in Figure 1 is likely to represent the whole of PDK1.

Human ESTs encoding PDK1 are derived from many tissues (Table 1). Interestingly, the nucleotide sequence

Table 1

GenBank accession numbers for PDK1 ESTs.

Accession number	Tissue from which EST derives
AA186323	HeLa cells
H97903	Melanocyte
AA018098	Retina
AA018097	Retina
AA019394	Retina
AA019393	Retina
N22904	Melanocyte
W94736	Fetal heart
EST51985	Gall bladder
N31292	Melanocyte
AA188174	HeLa cells
AA100210	Colon
R84271	Retina
AA121994	Pancreas

of PDK1 possessed 100% identity to the partial sequence of a gene that has been mapped to a 700 kb region on human chromosome 16p13.3 close to the genes responsible for polycystic kidney disease type 1 and tuberous sclerosis disease type 2 [22]. This gene is known to be expressed as an 8 kb transcript in heart, brain, placenta, lung, skeletal muscle, kidney and pancreas [22].

PDK1 is a human homologue of the *Drosophila* DSTPK61 protein kinase

The catalytic domain of PDK1 spans residues 84–341 (Figure 1) and contains all of the classical kinase subdomains I–XI [23]. It is most similar to the subfamily of protein kinases that include PKA (39% identity to the catalytic domain), PKC (36% identity to the ζ isoform) and

Figure 1

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                  CCGCTTCGGGGAGGAGGACGCTGAGGAGGCGCCGAGCCGCGCAGCGCTGCGGGGGAGGCGCCCGCGCCGACGCGGGGCCC
  1   ATGGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTATGTTCCTGCCCATCCCCATCAATGGTGAGG      90
  1    M  A  R  T  T  S  Q  L  Y  D  A  V  P  I  Q  S  S  V  V  L  C  S  C  P  S  P  S  M  V  R      30

 91   ACCCAGACTGAGTCCAGCACGCCCCCTGGCATTCCTGGTGGCAGCAGGCAGGGCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCC     180
 31    T  Q  T  E  S  S  T  P  P  G  I  P  G  G  S  R  Q  G  P  A  M  D  G  T  A  A  E  P  R  P      60

181   GGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGCCGCAGCCTCGGAAGAAGCGGCCTGAGGACTTCAAGTTTGGGAAAATCCTTGGGGAA     270
 61    G  A  G  S  L  Q  H  A  Q  P  P  P  Q  P  R  K  K  R  P  E  D  F  K  F  G  K  I  L  G  E      90

271   GGCTCTTTTTCCACGGTTGTCCTGGCTCGAGAACTGGCAACCTCCAGAGAATATGCGATTAAAATTCTGGAGAAGCGACATATCATAAAA     360
 91    G  S  F  S  T  V  V  L  A  R  E  L  A  T  S  R  E  Y  A  I  K  I  L  E  K  R  H  I  I  K     120

361   GAGAACAAGGTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTTAAGCTTTACTTCACATTTCAG     450
121    E  N  K  V  P  Y  V  T  R  E  R  D  V  M  S  R  L  D  H  P  F  F  V  K  L  Y  F  T  F  Q     150

451   GACGACGAGAAGCTGTATTTCGGCCTTAGTTATGCCAAAAATGGAGAACTACTTAAATATATTCGCAAAATCGGTTCATTCGATGAGACC     540
151    D  D  E  K  L  Y  F  G  L  S  Y  A  K  N  G  E  L  L  K  Y  I  R  K  I  G  S  F  D  E  T     180

541   TGTACCCGATTTTACACGGCTGAGATCGTGTCTGCTTTAGAGTACTTGCACGGCAAGGGCATCATTCACAGGGACCTTAAACCGGAAAAC     630
181    C  T  R  F  Y  T  A  E  I  V  S  A  L  E  Y  L  H  G  K  G  I  I  H  R  D  L  K  P  E  N     210

631   ATTTTGTTAAATGAAGATATGCACATCCAGATCACAGATTTTGGAACAGCAAAAGTCTTATCCCCAGAGAGCAAACAAGCCAGGGCCAAC     720
211    I  L  L  N  E  D  M  H  I  Q  I  T  D  F  G  T  A  K  V  L  S  P  E  S  K  Q  A  R  A  N     240

721   TCATTCGTGGGAACAGCGCAGTACGTTTCTCCAGAGCTGCTCACGGAGAAGTCCGCCTGTAAGAGTTCAGACCTTTGGGCTCTTGGATGC     810
241    S  F  V  G  T  A  Q  Y  V  S  P  E  L  L  T  E  K  S  A  C  K  S  S  D  L  W  A  L  G  C     270

811   ATAATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCTTATATTTCAGAAGATCATTAAGTTGGAATATGAC     900
271    I  I  Y  Q  L  V  A  G  L  P  P  F  R  A  G  N  E  Y  L  I  F  Q  K  I  I  K  L  E  Y  D     300

901   TTTCCAGAAAAATTCTTCCCTAAGGCAAGAGACCTCGTGGAGAAACTTTTGGTTTTAGATGCCACAAAGCGGTTAGGCTGTGAGGAAATG     990
301    F  P  E  K  F  F  P  K  A  R  D  L  V  E  K  L  L  V  L  D  A  T  K  R  L  G  C  E  E  M     330

991   GAAGGATACGGACCTCTTAAAGCACACCCGTTCTTCGAGTCCGTCACGTGGGAGAACCTGCACCAGCAGACGCCTCCGAAGCTCACCGCT    1080
331    E  G  Y  G  P  L  K  A  H  P  F  F  E  S  V  T  W  E  N  L  H  Q  Q  T  P  P  K  L  T  A     360

1081  TACCTGCCGGCTATGTCGGAAGACGACGAGGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCATGCAGGTGTCTTCG    1170
361    Y  L  P  A  M  S  E  D  D  E  D  C  Y  G  N  Y  D  N  L  L  S  Q  F  G  C  M  Q  V  S  S     390

1171  TCCTCCTCCTCACACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCTGGAC    1260
391    S  S  S  S  H  S  L  S  A  S  D  T  G  L  P  Q  R  S  G  S  N  I  E  Q  Y  I  H  D  L  D     420

1261  TCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAGAAGAGGTTGTTGTTGGAGAAGCAGGCTGGCGGAAACCCTTGGCACCAG    1350
421    S  N  S  F  E  L  D  L  Q  F  S  E  D  E  K  R  L  L  L  E  K  Q  A  G  G  N  P  W  H  Q     450

1351  TTTGTAGAAAATAATTTAATACTAAAGATGGGCCCAGTGGATAAGCGGAAGGGTTTATTTGCAAGACGACGACAGCTGTTGCTCACAGAA    1440
451    F  V  E  N  N  L  I  L  K  M  G  P  V  D  K  R  K  G  L  F  A  R  R  R  Q  L  L  L  T  E     480

1441  GGACCACATTTATATTATGTGGATCCTGTCAACAAAGTTCTGAAAGGTGAAATTCCTTGGTCACAAGAACTTCGACCAGAGGCCAAGAAT    1530
481    G  P  H  L  Y  Y  V  D  P  V  N  K  V  L  K  G  E  I  P  W  S  Q  E  L  R  P  E  A  K  N     510

1531  TTTAAAACTTTCTTTGTCCACACGCCTAACAGGACGTATTATCTGATGGACCCCAGCGGGAACGCACACAAGTGGTGCAGGAAGATCCAG    1620
511    F  K  T  F  F  V  H  T  P  N  R  T  Y  Y  L  M  D  P  S  G  N  A  H  K  W  C  R  K  I  Q     540

1621  GAGGTTTGGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGTGACGTGGCCTGCGGCCGGGCTGCCCTTCGCTGCCAGGACAC    1710
541    E  V  W  R  Q  R  Y  Q  S  H  P  D  A  A  V  Q  *                                            556

1711  CTGCCCCAGCGCGGCTTGGCCGCCATCCGGGACGCTTCCAGACCACCTGCCAGCCATCACAAGGGGAACGCAGAGGCGGAAACCTTGCAG    1800
1801  CATTTTTATTT                                                                                  1811
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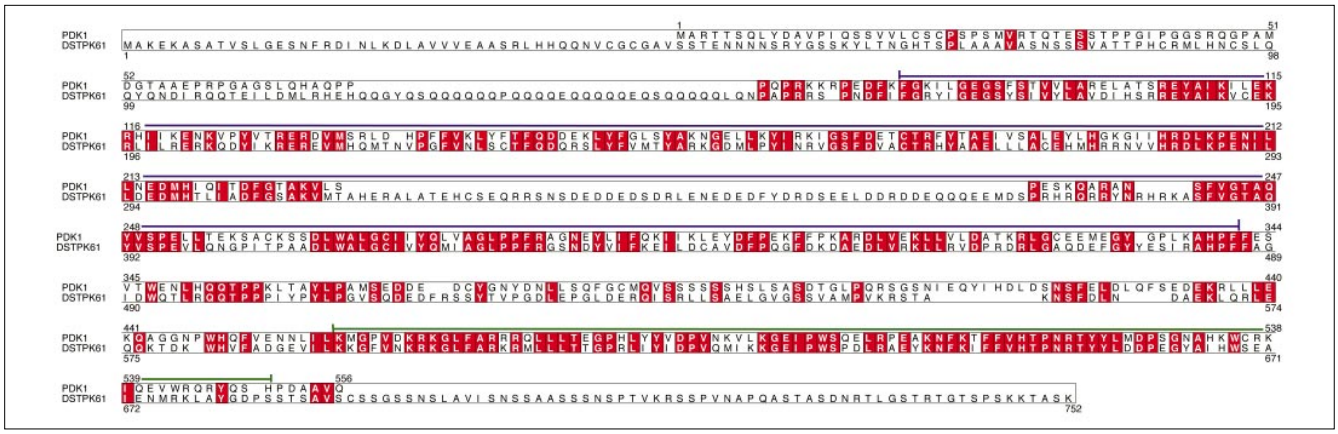
Nucleotide and deduced amino acid sequence of human PDK1. The amino acid sequences that are underlined correspond to the sequences of tryptic peptides isolated from PDK1 purified from rabbit skeletal muscle. The kinase catalytic domain lies between residues 84 and 341 and the putative PH domain lies between residues 459 and 550. The asterisk denotes the stop codon in the DNA sequence. The GenBank accession number for PDK1 is AF017995.

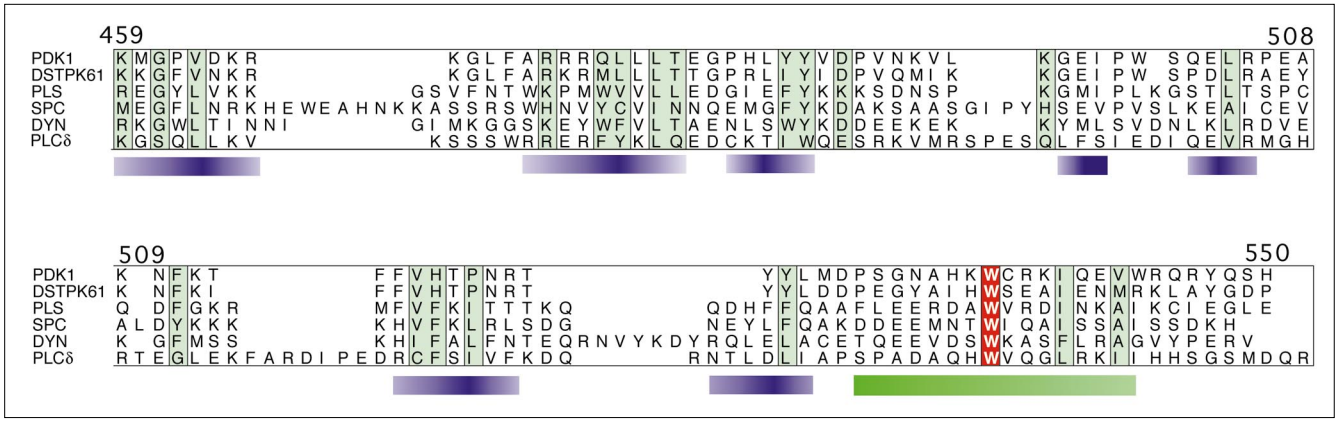
Figure 2



Alignment of the amino acid sequences of human PDK1 and *Drosophila* DSTPK61. The alignment was carried out using the Clustal W program [46]. The catalytic domain is shown by a solid blue line and comprises residues 84–341 of PDK1 and residues 164–486 of DSTPK61. The putative PH domain is shown by a solid green line and lies between residues 459 and 550 of PDK1 and 592 and 684 of DSTPK61. Identical amino acids are shown in red.

PKBα (35% identity). Although the PDK1 catalytic domain possessed similarity to many protein kinases of the PKA subfamily, only one of these showed homology to PDK1 in the noncatalytic region: the *Drosophila* serine/threonine protein kinase-61 (DSTPK61; GenBank accession number Y07908; C.M., M. Todman and M.B., unpublished observations), which was not only 54% identical to PDK1 in the catalytic domain, but was also very similar in the noncatalytic carboxy-terminal domain (Figure 2). This homology was most striking between residues 450 and 550 of PDK1, where the identity to DSTPK61 was 61% (79% similarity). These observations suggested that DSTPK61 might be a *Drosophila* homologue of PDK1. DSTPK61 contains a 60-residue carboxy-terminal extension not found in PDK1 and there is little homology between the amino-terminal 70 residues of PDK1 and the amino-terminal 150 residues of DSTPK61 (Figure 2). PDK1 also lacks the polyglutamine insertion near the amino terminus of DSTPK61 (residues 120–150) and a further polyacidic insertion located between subdomains VII and VIII (residues 312–370) of the kinase domain (Figure 2). The physiological role of DSTPK61 is considered further in the Discussion section.

Figure 3



Evidence that PDK1 and DSTPK61 possess a PH domain: sequence alignment of PH domains that have a known tertiary structure with the putative PH domains of PDK1 and DSTPK61. Alignment was done using the program AMPS [47] and formatted using AMAS [48] with some manual adjustment to ensure coincidence of secondary structure regions. Regions containing conserved residues are coloured green and the invariant tryptophan (Trp535) is coloured red. General regions of secondary structure are denoted by the blue (β-strand) and green (α-helix) bands. The PH sequences are those found in human pleckstrin (PLS), human spectrin (SPC), human dynamin (DYN), and rat phospholipase C-δ (PLCδ). Numbering is based on the PDK1 sequence (see Figure 1).

PDK1 and DSTPK61 possess a carboxy-terminal pleckstrin homology domain

The high level of sequence conservation between PDK1 and DSTPK61, from residues 450–550 of PDK1 (Figure 2), suggested that this region is likely to have an important function. Inspection of these sequences indicated that they are likely to be PH domains. These domains of ~100 residues, which have been found in over 70 other proteins, are predicted to fold into similar three-dimensional structures and may mediate protein–lipid interactions, protein–protein interactions, or both [24,25]. We have performed sequence alignments of residues 459–550 in PDK1 and DSTPK61 with the PH domains of pleckstrin, spectrin, dynamin and phospholipase C-δ, whose tertiary structures have been determined (Figure 3), and with 71 other PH domain sequences (data not shown). Although the sequence identities between PH domains in general are poor [24,25], there are certain residues that show a high level of conservation. For PDK1 and DSTPK61, a single position, namely Trp535 of PDK1 (Figure 3), is invariant throughout the PH domain family. Many similar amino acids are found at defined regions of the PH domain (Figure 3) [24,25]. Secondary structure predictions also indicate that residues 459–525 of PDK1 are likely to contain regions of β-sheet, while residues 528–542 (Figure 3) are likely to form an extended α-helix, a prediction that is consistent with the known structures of other PH domains [24,25].

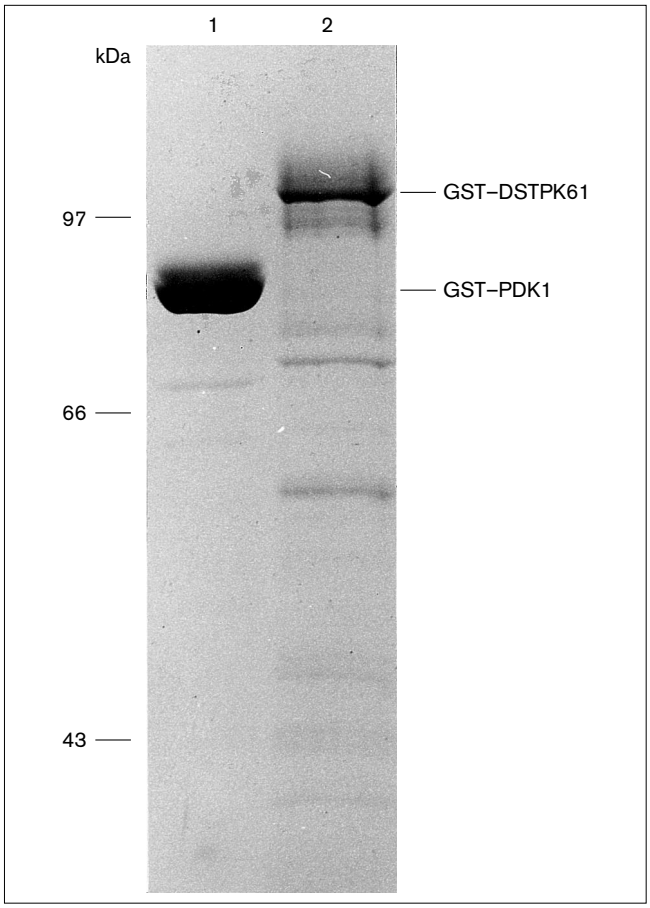
Expression of GST–PDK1 and GST–DSTPK61

Residues 52–556 of PDK1 were expressed in human embryonic kidney 293 cells and residues 1–752 of DSTPK61 in *Escherichia coli* as fusion proteins with GST at the amino terminus (hereafter termed GST–PDK1 and GST–DSTPK61), and both were purified on glutathione-Sepharose. The GST–PDK1 preparation showed two bands with apparent molecular masses of 87 kDa and 85 kDa (Figure 4, Lane 1) and 2 mg of purified GST–PDK1 protein were obtained from 20 (10 cm diameter) dishes of cells. The GST–DSTPK61 preparation showed a diffuse band at the expected molecular mass (105 kDa) together with a number of minor degradation products (Figure 4, Lane 2): 0.35 mg of GST–DSTPK61 were obtained from 0.5 l of bacterial culture.

GST–PDK1 and GST–DSTPK61 activate and phosphorylate PKBα in a PtdIns(3,4,5)P_3- or PtdIns(3,4)P_2-dependent manner

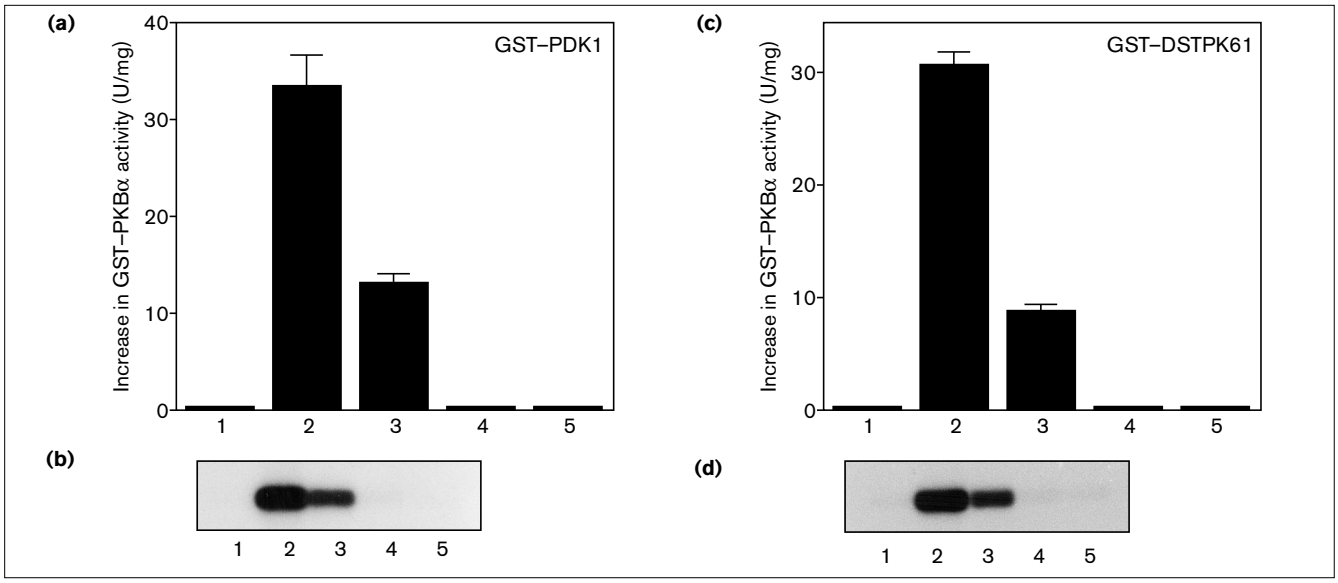
GST–PDK1 and GST–DSTPK61 both activated and phosphorylated GST–PKBα in the presence of a vesicle background containing phosphatidylcholine (PtdCho) and phosphatidylserine (PtdSer), provided that PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 were included. The extent of activation of GST–PKBα correlated with the extent of phosphorylation (Figure 5), and no activation or phosphorylation occurred if PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 were replaced by either PtdIns(4,5)P_2 or PtdIns3-P (Figure 5). Identical results were obtained using purified PDK1 from rabbit skeletal muscle and full-length GST–PDK1 (residues 1–556) expressed in 293 cells (data not shown). A catalytically inactive GST–PDK1 mutant in which Asp223 was changed to alanine did not phosphorylate or activate GST–PKBα in the presence of PtdCho/PtdSer lipid vesicles containing PtdIns(3,4,5)P_3 (data not shown). The specific activities of GST–PDK1 (residues 52–556, 78,000 U/mg) and GST–PDK1 (residues 1–556, 89,000 U/mg) towards GST–PKBα in the presence of PtdIns(3,4,5)P_3 were similar to PDK1 purified from rabbit skeletal muscle (100,000 U/mg), being over 200 times higher than that of bacterially expressed GST–DSTPK61 (280 U/mg). Further work is needed to establish whether this difference is caused by misfolding of DSTPK61 or lack of an important

Figure 4



SDS–polyacrylamide gel of purified GST–PDK1 and GST–DSTPK61. GST–PDK1 residues 52–556 (lane 1; 3 μg protein) and GST–DSTPK61 (lane 2; 6 μg protein) were electrophoresed on a 7.5% 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. The other GST–PDK1 constructs used in this study were all expressed at a similar level of purity as the GST–PDK1 preparation shown in lane 1 (data not shown).

Figure 5



GST–PDK1 and GST–DSTPK61 activate and phosphorylate PKBα in a PtdIns(3,4,5)P_3- or PtdIns(3,4)P_2-dependent manner. GST–PKBα was incubated for 30 min at 30°C with 1 unit/ml **(a,b)** GST–PDK1 (residues 52–556) or **(c,d)** GST–DSTPK61 with Mg[γ^{32}P]ATP and phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer, and various PtdIns lipids (numbered 1–5, see below) all at a final concentration of 10 μM in the assay. Under these conditions, the increase in PKBα activity and phosphorylation was linear with respect to time (see Materials and methods). In (a) and (c), the assays were terminated by making the incubations 1% (by volume) in Triton X-100 (see [21]), and the increase in specific activity of GST–PKBα was determined. In (b) and (d), the reactions were terminated by making the solutions 1% in SDS, the samples were subjected to SDS–polyacrylamide gel electrophoresis, and the phosphorylation was assessed by autoradiography of the Coomassie blue stained band corresponding to GST–PKBα. The results are presented in (a) and (c) as the increase in the specific activity of GST–PKBα (U/mg), relative to a control incubation in which GST–PDK1 or GST–DSTPK61 was omitted (± SEM for 6–9 determinations, three independent experiments). The basal activity of GST–PKBα was 2.5 U/mg. Lane 1, buffer control; lane 2, *sn*-1-stearoyl, 2-arachidonyl D-PtdIns(3,4,5)P_3; lane 3, *sn*-1,2-dipalmitoyl D-PtdIns(3,4)P_2; lane 4, PtdIns(4,5)P_2 (purified from Folch brain fraction); lane 5, *sn*-1,2-dipalmitoyl D-PtdIns-3P.

post-translational modification when it is expressed in *E. coli*, or whether DSTPK61 does not recognise human PKBα as well as human PDK1. Some activation and phosphorylation of GST–PKBα was obtained using very high concentrations of GST–PDK1 or GST–DSTPK61 in the absence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. However, the activity of PDK1 was enhanced about 1000-fold in the presence of these inositol phospholipids. In the presence of PtdIns(3,4,5)P_3 and at high concentrations of GST–PDK1 and GST–DSTPK61, the phosphorylation of GST–PKBα reached 0.75 moles of phosphate per mole of protein and was paralleled by a 35-fold increase in activity. This was similar to the maximal activation of GST–PKBα obtained using PDK1 from skeletal muscle [21].

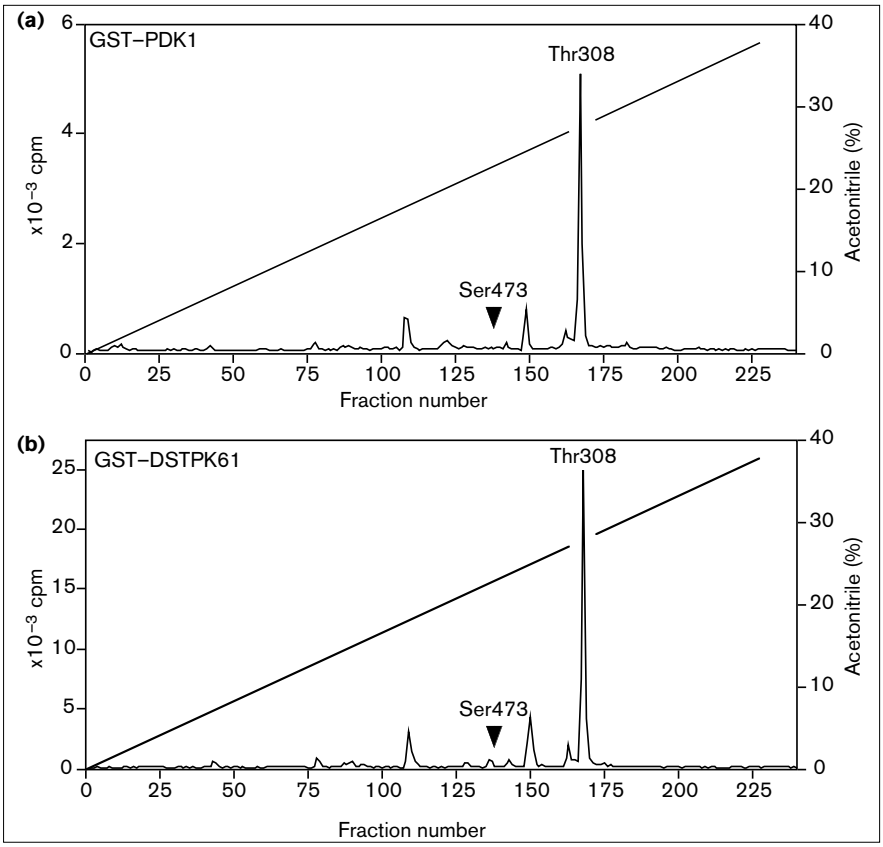
Expressed PDK1 and DSTPK61 phosphorylate PKBα at Thr308

^{32}P-labelled GST–PKBα that had been maximally phosphorylated with either GST–PDK1 (Figure 6a) or GST–DSTPK61 (Figure 6b) was digested with trypsin and chromatographed on a C_{18} column. One major ^{32}P-labelled peptide was obtained in each case. This peptide eluted at 26% acetonitrile at the same position as the ^{32}P-labelled PKBα tryptic phosphopeptide comprising residues 308–327 [20,21]. This peptide contained phosphothreonine, and when subjected to solid phase sequencing, ^{32}P-radioactivity was released after the first cycle of Edman degradation, confirming Thr308 as the site of phosphorylation (data not shown).

PKBα is activated by cotransfection with PDK1 in 293 cells and phosphorylated at Thr308

In order to determine whether PDK1 was capable of activating PKBα in a cellular context, we transfected a haemagglutinin epitope-tagged PKBα (HA–PKBα) into 293 cells, either alone or together with Myc epitope-tagged PDK1 (Myc–PDK1). As reported previously, HA–PKBα possessed a low basal activity when transfected alone into 293 cells, which was increased 40-fold after stimulation with IGF1 (Figure 7) [20]. When 293 cells were transfected with both HA–PKBα and Myc–PDK1, however, the activity of HA–PKBα was increased 20-fold in unstimulated cells and 70-fold after stimulation with IGF1 (Figure 7). When 293 cells were transfected with HA–PKBα and a catalytically inactive mutant of PDK1 (Myc–D223A-PDK1), HA–PKBα was not activated significantly (Figure 7).

Figure 6



PDK1 phosphorylates PKBα at Thr308. GST–PKBα was maximally phosphorylated by incubation for 30 min with 25 U/ml of either **(a)** GST–PDK1 (residues 52–556) or **(b)** GST–DSTPK61 and phospholipid vesicles comprising PtdCho, PtdSer, the D-enantiomer of 1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 and Mg–γ[^{32}P]-ATP (10^6 cpm/nmol). After 30 min, the samples were alkylated with 4-vinylpyridine, digested with trypsin [21] and applied to a Vydac 218TP54 C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. 38% (a) and 45% (b) of the radioactivity applied to the column was recovered in the major ^{32}P-containing peptide eluting at 26% acetonitrile, which contains Thr308. The elution position of the tryptic peptide containing Ser473 is also marked (see [20] and Figure 8).

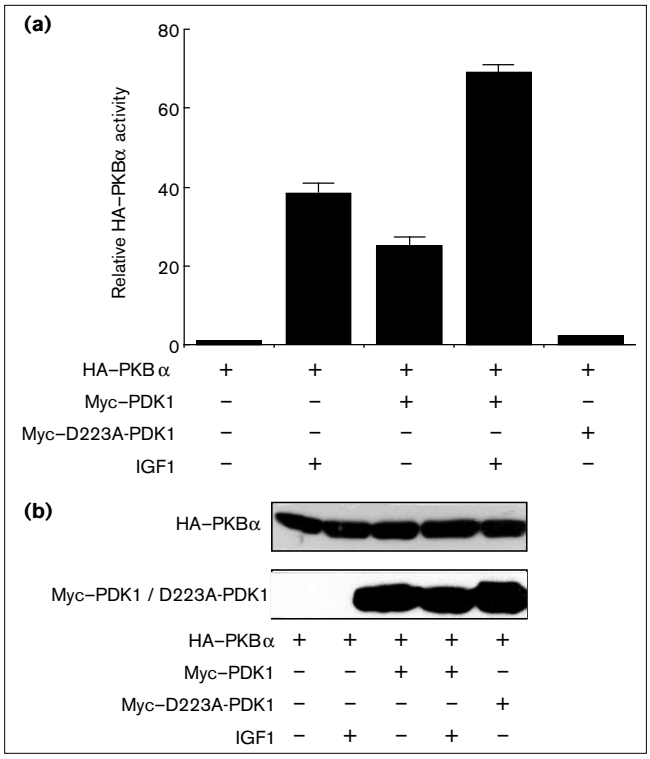
In order to establish the mechanism by which overexpression of PDK1 in 293 cells induced the activation of PKBα, the cells were incubated with ^{32}P-phosphate, stimulated with buffer or IGF1 and ^{32}P-labelled HA-PKBα was immunoprecipitated from the lysates. After digestion with trypsin, the resulting peptides were analysed by C_{18} chromatography (Figure 8). As observed previously [20], HA–PKBα is phosphorylated at Ser124 and Thr450 in unstimulated cells, and IGF1 stimulation induces the phosphorylation of Thr308 and Ser473 (Figure 8). In contrast, when cotransfected with PDK1, HA–PKBα became partially phosphorylated at Thr308 in unstimulated 293 cells, to a level that was 70% of that observed in IGF1-stimulated cells transfected with HA–PKBα alone. This phosphorylation of Thr308 was increased a further fourfold in response to IGF1 (Figure 8). Importantly, cotransfection of Myc–PDK1 with HA–PKBα did not induce phosphorylation of HA–PKBα at Ser473 in unstimulated cells, nor did it potentiate the level of Ser473 phosphorylation following IGF1 stimulation (Figure 8).

Role of the PH domain in the activation of PKBα by PDK1

A GST–PKBα mutant lacking the PH domain (GST–ΔPH-PKBα, residues 118–479) possessed a threefold higher basal activity than that of full-length wild-type GST–PKBα and was activated (Figure 9a) and phosphorylated (data not shown) by PDK1 in a PtdIns(3,4,5)P_3-independent manner. The rate of activation, however, was reduced about 20-fold compared to wild-type GST–PKBα (Figure 9a). PDK1 purified from rabbit skeletal muscle extracts also activated and phosphorylated GST–ΔPH-PKBα in a PtdIns(3,4,5)P_3-independent manner (data not shown).

A PDK1 mutant lacking the putative carboxy-terminal PH domain was expressed as a GST fusion protein in 293 cells (GST–ΔPH-PDK1, residues 1–450). We found that this form of PDK1 was still able to activate GST–PKBα in a PtdIns(3,4,5)P_3-dependent manner, but the rate of activation was reduced about 30-fold compared to full-length wild-type GST–PDK1 (Figure 9b). As observed with PDK1 purified from rabbit skeletal muscle [21] or full-length GST–PDK1 (data not shown), GST–ΔPH-PDK1 was activated more effectively by the D-enantiomer of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 than by the D-enantiomer of *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3, and no activation was induced by the L-enantiomer of *sn*-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 (Figure 9b). In contrast, *sn*-1,2-dipalmitoyl D-PtdIns(3,4)P_2 was very poor at inducing the activation of GST–PKBα by GST–ΔPH-PDK1 (Figure 9b) under conditions where this lipid was

Figure 7



PKBα is activated by cotransfection with PDK1 in 293 cells. **(a)** 293 cells were transiently transfected with DNA constructs expressing either HA–PKBα or both HA–PKBα and Myc–PDK1 (residues 52–556) or a catalytically inactive Myc–PDK1 (Myc–D223A-PDK1, residues 1–556). The cells were then stimulated for 10 min with or without 100 ng/ml IGF1, the HA–PKBα immunoprecipitated from the lysates and assayed. The results are expressed relative to the specific activity of wild-type HA–PKBα in unstimulated 293 cells (0.5 ± 0.03 U/mg). No PKBα activity was detected in mock transfections in which the cells were transfected with PCMV5 vector alone (data not shown). **(b)** Protein (6 μg) from each lysate was electrophoresed on a 10% SDS–polyacrylamide gel and immunoblotted using either a monoclonal HA antibody or Myc antibody to detect HA–PKBα or Myc–PDK1, respectively.

as effective as *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3 at stimulating GST–PKBα activation by either full-length GST–PDK1 (data not shown) or PDK1 purified from rabbit skeletal muscle [21].

PDK1 is not activated or phosphorylated by IGF1

293 cells were serum starved for 16 hours, stimulated with IGF1, lysed and the endogenous PDK1 activity present in the cell lysates was determined after chromatography on heparin–Sepharose (see Materials and methods). IGF1 stimulation of cells for up to 10 minutes did not result in any activation or inhibition of PDK1 activity (data not shown).

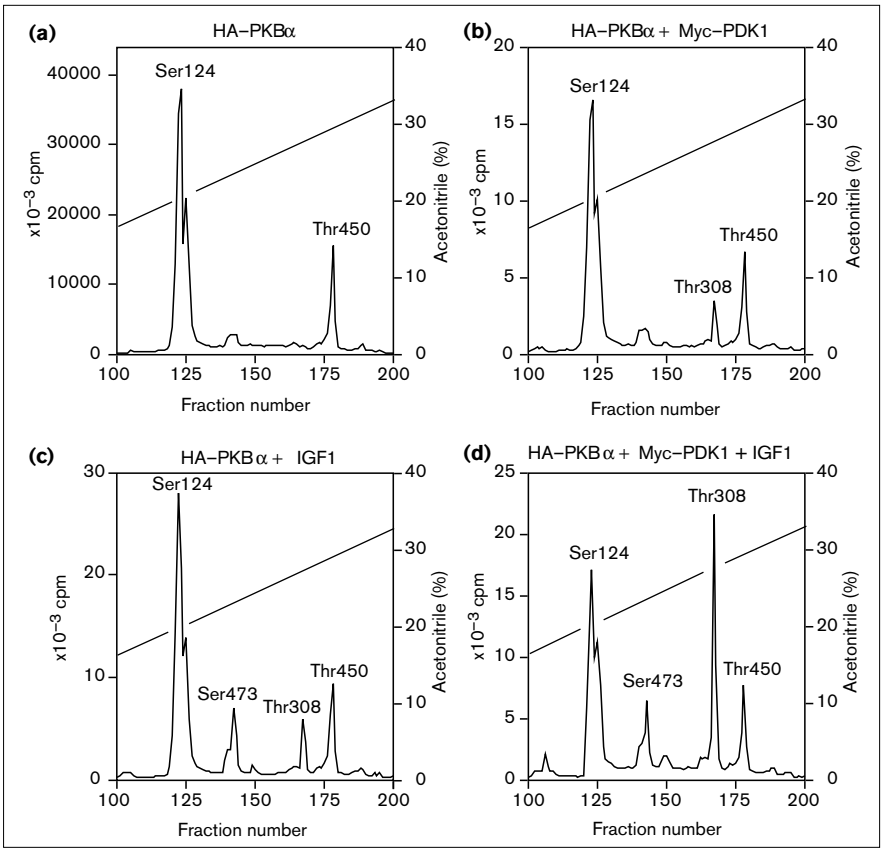
In order to see whether IGF1 stimulation was inducing the phosphorylation of PDK1, 293 cells were transfected with Myc–PDK1 (residues 52–556), incubated with ^{32}P-phosphate, stimulated with buffer or IGF1 for 5 minutes and ^{32}P-labelled Myc–PDK1 was immunoprecipitated from the lysates. After digestion with trypsin, the resulting peptides were analysed by C_{18} chromatography. These experiments demonstrate that PDK1 is phosphorylated at four tryptic peptides (only on serine residues) in unstimulated cells and that IGF1 does not alter the phosphorylation of any of these peptides (data not shown). Treatment of PDK1 purified from rabbit skeletal muscle with high concentrations of the serine/threonine-specific protein phosphatase 2A and protein tyrosine phosphatase 1B also had no effect on activity (data not shown). One of the *in vivo* phosphorylation sites on PDK1 was identified as Ser241, which lies in the equivalent position to the Thr308 of PKB in the kinase domain.

Discussion

PDK1 purified 500,000-fold from skeletal muscle [21] contains three proteins with apparent molecular masses of 85 kDa, 45 kDa and 67–69 kDa and, in this paper, we have established that the 67–69 kDa component is PDK1. When cloned and expressed in 293 cells, this protein activated PKBα and induced its phosphorylation at Thr308, both activation and phosphorylation being dependent on PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. When overexpressed in 293 cells, PDK1 also activated PKBα in cotransfection experiments and potentiated the phosphorylation of Thr308 by IGF1. These observations and the finding that, like PKB, the mRNA encoding PDK1 is expressed ubiquitously (Table 1) are consistent with PDK1 lying 'upstream' of PKBα *in vivo*.

It is of interest that PDK1 lies in the same subfamily of protein kinases as PKB. Moreover, like PKB, it also contains a PH domain although, in contrast to PKB, this is located carboxy-terminal to the catalytic domain [13]. The PH domain in PKBα is capable of binding PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at micromolar concentrations [26,27], which may facilitate its translocation to the plasma membrane, which occurs in response to IGF1 in 293 cells (M. Andjelkovic *et al.*, unpublished observations) or interleukin-2 stimulation of EL4·IL-2 cells [28] to form a signalling complex ([21]; M. Andjelkovic *et al.*, unpublished observations). However, the finding presented in this paper that PDK1 is not activated by IGF1, and that a mutant of PKBα lacking the PH domain is activated and phosphorylated independently of PtdIns(3,4,5)P_3 (Figure 9a), demonstrates that the PtdIns(3,4,5)P_3-induced activation of PKBα by PDK1 is substrate directed, at least in part. It is possible that the binding of PtdIns(3,4,5)P_3 to the PH domain of PKBα alters the conformation of PKBα so that Thr308 becomes accessible for phosphorylation by PDK1. Consistent with this model, deletion of the PH domain of PDK1 resulted in an enzyme that is still able to activate and phosphorylate PKBα in a PtdIns(3,4,5)P_3-dependent manner (Figure 9b).

Figure 8



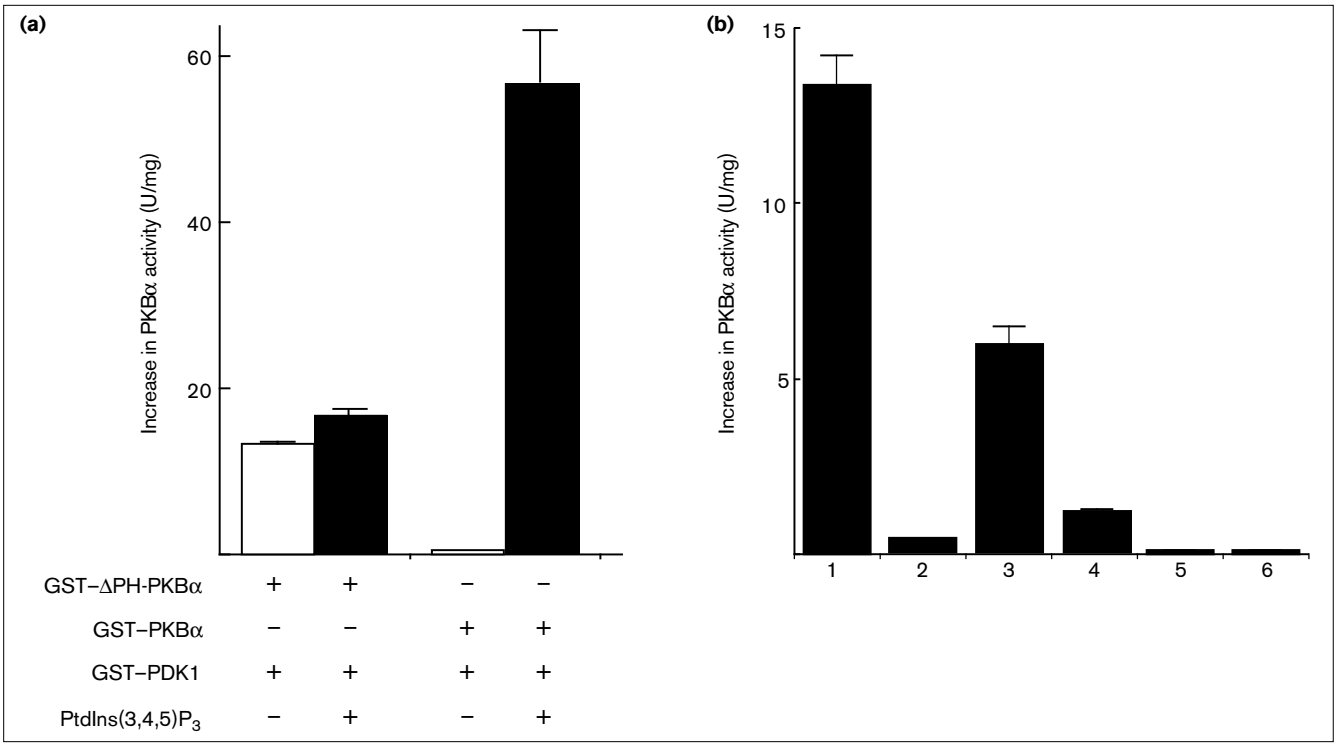
Overexpression of PDK1 induces phosphorylation of PKBα at Thr308. 293 cells transiently transfected with either **(a,c)** wild-type HA–PKBα or **(b,d)** HA–PKBα and Myc–PDK1 (residues 52–556) were incubated with ^{32}P-orthophosphate, then treated for 10 min without (a,b) or with (c,d) 100 ng/ml IGF1. The ^{32}P-labelled HA–PKBα was immunoprecipitated from the lysates, treated with 4-vinylpyridine and electrophoresed on a 10% polyacrylamide gel. The HA–PKBα was excised from the gel, digested with trypsin and chromatographed on a C_{18} column (see Figure 6 legend) to resolve the four major phosphopeptides that are phosphorylated at Ser124, Ser473, Thr308 and Thr450, respectively [20]. The identity of each phosphopeptide was established by phospho-amino acid analysis and solid phase sequencing. Similar results were obtained in two separate experiments for (a,c) and in three separate experiments for (b,d).

The rate of activation of PKBα by PDK1 is, however, reduced 30-fold when the PH domain of PDK1 is deleted. Thus, it remains possible that the PH domain of PDK1 binds PtdIns(3,4,5)P_3 and that this greatly enhances the rate of activation of PKBα. PtdIns(3,4,5)P_3 can stimulate the activation of PKBα by PDK1 only when it is presented in lipid vesicles containing PtdCho and PtdSer [21], and a PDK1 mutant lacking the PH domain may not be able to interact with lipids and hence be unable to penetrate these vesicles efficiently. This may account for the greatly reduced rate at which PDK1 lacking the PH domain activates PKBα. PKBα lacking the PH domain is also presumably unable to penetrate lipid vesicles and this may explain why this mutant is phosphorylated at a 20-fold lower rate by GST–PDK1 than full-length PKBα (Figure 9a). It is clearly critical to study the interaction of PDK1 with 3-phosphoinositides.

Recently, an enzyme with very similar properties to PDK1 has been partially purified from COS-1 and brain cell cytosol extracts [29]. Like PDK1, this kinase phosphorylated Thr308 of PKBα only in the presence of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 and not PtdIns(4,5)P_2 lipids. Furthermore, this activity was found not to phosphorylate PKBα mutants that could not interact with PtdIns(3,4,5)P_3. This finding is consistent with the idea that interaction of PtdIns(3,4,5)P_3 with the PH domain of PKBα is required to permit phosphorylation of Thr308 of PKBα by upstream kinases. In contrast to our work with purified PDK1, the kinase from brain cytosol phosphorylation of ΔPH-PKBα was still activated three-fold by the addition of PtdIns(3,4,5)P_3, whereas PDK1 phosphorylation of ΔPH-PKBα is not significantly activated by PtdIns(3,4,5)P_3 (Figure 9). It will be important to establish whether the activity described in [29] is actually PDK1 or another enzyme capable of activating PKBα by phosphorylating Thr308.

Although PKBα is activated by cotransfection with PDK1 in 293 cells, and phosphorylation of PKBα at Thr308 induced by IGF1 is potentiated, the overexpression of PDK1 does not potentiate the activation of PKBα by IGF1 (Figure 7). The reason is that the activation of PKBα also requires phosphorylation of Ser473, and phosphorylation of Thr308 and Ser473 have synergistic effects on activity [20]. An important finding made in the present study is that PKBα does not become phosphorylated at Ser473 when cotransfected with PDK1, and nor does transfection with PDK1 affect the level of Ser473 phosphorylation attained after stimulation with IGF1. These

Figure 9



Role of the PH domains of PKBα and PDK1. **(a)** 0.5 μg of either GST–PKBα or GST–ΔPH-PKBα were incubated for 30 min at 30°C with 2.3 nM GST–PDK1 (residues 1–556) with Mg[γ^{32}P]ATP and phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer either in the presence or absence of 10 μM PtdIns(3,4,5)P_3 in a 20 ml assay. The assays were terminated by making the incubations 1% (by volume) in Triton X-100 [21], and the increase in specific activity of GST–PKBα was determined. The basal activity of GST–PKBα was 2.5 U/mg and that of GST–ΔPH-PKBα was 8 U/mg. At the high concentration of GST–PDK1 used in this assay, the rate of activation of full-length wild-type GST–PKBα is not linear with time, whereas the activation of GST–ΔPH-PKBα is linear. When the experiments are carried out at 20-fold lower concentrations of GST–PDK1, a similar extent of activation of wild-type GST–PKBα is achieved as that shown in the figure for GST–ΔPH-PKBα by 2.3 nM GST–PDK1. **(b)** As in (a) except that 0.5 μg of GST–PKBα were incubated with 2.5 nM GST–ΔPH-PDK1 (residues 1–450) in the presence of phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer and 10 μM of either *sn*-1-stearoyl, 2-arachidonyl D-PtdIns(3,4,5)P_3 (lane 1); *sn*-1-stearoyl, 2-arachidonyl L-PtdIns(3,4,5)P_3 (lane 2), *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3 (lane 3), *sn*-1,2-dipalmitoyl D-PtdIns(3,4)P_3 (lane 4), PtdIns(4,5)P_2 (purified from Folch brain fraction; lane 5) or *sn*-1,2-dipalmitoyl D-PtdIns-3P (lane 6). The results are presented as the increase in the specific activity of GST–PKBα (U/mg; ± SEM for 6–9 determinations, three independent experiments), relative to a control incubation in which GST–PDK1 is omitted.

experiments demonstrate that the protein kinase that phosphorylates PKBα at Ser473 is not activated by PDK1, and that phosphorylation of PKBα at Thr308 in a cellular context does not cause PKBα to autophosphorylate at Ser473. Like the phosphorylation of Thr308, Ser473 phosphorylation is prevented by incubating cells with wortmannin prior to stimulation with insulin or IGF1. It will be interesting to know whether the Ser473 kinase is activated directly by PtdIns(3,4,5)P_3 or whether the binding of PtdIns(3,4,5)P_3 to PKBα alters its conformation to permit phosphorylation by the Ser473 kinase.

PDK1 was found to be homologous to DSTPK61, a *Drosophila* protein kinase that has been implicated in the regulation of sex differentiation, oogenesis and spermatogenesis (C.M., M. Todman and M.B., unpublished observations). The identity between PDK1 and DSTPK61 was 54% in the catalytic domain, but even greater (61% identity) in the putative PH domain (Figure 2), further suggesting an important functional role for the PH domain of PDK1. DSTPK61 expressed in *E. coli* activated human PKBα and phosphorylated it specifically at Thr308 in a PtdIns(3,4,5)P_3- or PtdIns(3,4)P_2-dependent manner (Figure 5). These findings indicate that DSTPK61 is a *Drosophila* homologue of PDK1 and suggest that one of its roles may be to activate the *Drosophila* PKB (termed DPKB), which was identified several years ago [30,31]. Although the physiological roles of DSTPK61 in *Drosophila* are unknown, the findings presented in this paper suggest that DPKB may lie downstream of DSTPK61 in signalling pathways regulating sex differentiation and oogenesis (C.M., M. Todman and M.B., unpublished observations). Since PtdIns 3-kinases play a major role in the activation of PKB in mammalian cells [4–6,13],

it is likely that a *Drosophila* PtdIns 3-kinase also plays an important role in the activation of DSTPK61 and DPKB. Little is known about the role of PtdIns 3-kinase in *Drosophila* [32]. However, a *Drosophila* PtdIns 3-kinase catalytic subunit termed Dp110 has been overexpressed in the wing and eye imaginal discs of *Drosophila*, in a normal, a constitutively active and a dominant-negative form [33]. The results of these studies showed that PtdIns 3-kinase is likely to play a role in regulating cell growth [34]. Interestingly, loss-of-function mutations in the *Drosophila* insulin receptor (Inr) also inhibited cell growth in imaginal discs and resulted in flies that were much smaller than wild type [34]. Taken together these results suggest that *Drosophila* PtdIns 3-kinases might be important targets of the insulin receptor [32] and, perhaps by acting through DSTPK61 and DPKB, may regulate cell growth.

The DSTPK61 gene is differentially spliced in male and female *Drosophila* (C.M., M. Todman and M.B., unpublished observations). This generates many different transcripts that all have the same open reading frame and, therefore, produce the same protein, but differ significantly in their 5′ and 3′ untranslated regions. This is likely to result in different levels of expression of the DSTPK61 protein in male and female *Drosophila* (C.M., M. Todman and M.B., unpublished observations). In view of the role of PKB in regulating apoptosis in mammalian cells (see Background), it is possible that the function of DSTPK61 during oogenesis, spermatogenesis and in female adults in relation to sex determination might be related to cell survival or cell death decisions. One could imagine the low level of the non-sex-specific transcript of DSTPK61 being responsible for a general growth function consistent with the role of overexpression of Dp110 in cells [33] and the specifically spliced forms seen in female carcasses and ovaries and male testes being controlled translationally, leading to the generation of high levels of protein at specific times.

It should be remembered that the extent and duration of activation of the MAP kinase cascade is critical in determining whether a signal induces the proliferation of PC12 cells or their differentiation to a sympathetic neurone like phenotype [35,36]. Similarly, different levels of expression of DSTPK61 are likely to affect the extent and duration of activation of PKB *in vivo* and hence may determine the precise function of this kinase cascade. There are two signalling pathways involved in sex differentiation in which such a situation may operate. The first involves the development of a specific adult male muscle in the abdomen required for holding the female fly during mating [37]. This muscle develops in males only in response to an unknown signal generated by adjacent nerve cells. It is likely that a signalling pathway causes muscle cells in that region of the female fly to apoptose, whereas in male flies the growth of these muscle cells is promoted [37]. The second pathway involves an unknown signalling mechanism that operates between the somatic cells and germ cells in the male and female gonads, in which a signal derived from the somatic cells initiates a signalling pathway that promotes survival of germline cells and induces these to differentiate into sperm and oocytes [38,39]. The mediators of both of these signalling pathways are at present unknown but, in light of the findings presented in this paper, it is possible that the unknown signals induce the sequential activation of PtdIns 3-kinase, DSTPK61 and DPKB. The role of this pathway could then be to mediate cell survival by inhibiting apoptosis. To understand the function of these signalling pathways, future work will have to concentrate on obtaining mutants of the *Drosophila* DSTPK61, PKB and PtdIns 3-kinase genes, as well as identifying the specific signals that switch on these pathways. It would be of considerable interest to compare the effects of expressing constitutively active and dominant-negative forms of Inr, Dp110, DSTPK61 and DPKB during oogenesis, spermatogenesis and sex-specific muscle differentiation.

Materials and methods

Materials

All phospholipids were obtained from the sources described previously [21]. Glutathione-Sepharose, the pGEX-3X expression vector and 1 ml heparin-Sepharose columns (HiTrap) were purchased from Pharmacia (Milton Keynes, UK), monoclonal 12CA5 and 9E10 antibodies from Boehringer Mannheim (Lewes, UK), alkylated trypsin and the pSP72 cloning vector from Promega (Southampton, UK), and the pCR 2.1-TOPO cloning vector from Invitrogen (Leek, Netherlands). GST–PKBα and sources of all other materials are described in [21]. The GenBank accession number for PDK1 is AF017995.

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 μM benzamidine, 0.2 μM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml 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 sodium phosphate pH 7.5 and 0.15 M NaCl.

Tryptic digestion of PDK1 and amino acid sequencing

PDK1 purified from 500 g of skeletal muscle [21] was denatured in 1% (by mass) SDS, electrophoresed on a 7.5% SDS–polyacrylamide gel and stained with Coomassie blue. After destaining, gel pieces containing the 85 kDa, 45 kDa and 67–69 kDa bands (~0.5 μg protein per band) were excised, digested with trypsin and the resulting peptides chromatographed on a Vydac C_{18} column and sequenced [40].

Cloning of PDK1

Most of the open reading frame of PDK1 was derived by interrogation of the dbEST database at the National Centre for Biological Information (http://www.ncbi.nlm.nih.gov/dbEST/index.html). A full-length cDNA clone for PDK1 was isolated by hybridisation screening of a cDNA library, in the vector λZAP, made from the human breast cancer cell line MCF7 (a gift of P. Mitchell, Institute of Cancer Research, Sutton, UK). The PDK1 probe for the screening was generated by RT-PCR with the primers CTGAGCCAGTTTGGCTGC and ACGTCCTGTTAGGCGTGTGG, corresponding to nucleotides 1138–1567 of the PDK1 sequence, with MCF7 cDNA as template. DNA sequencing was carried out on an Applied Biosystems 373A DNA automatic sequencer using the Taq dye terminator cycle sequencing kit.

Preparation of DNA expression constructs encoding PDK1 and GST–DSTPK61

Two overlapping human EST cDNA clones encoding PDK1 (GenBank accession numbers AA121994 and AA186323), corresponding to nucleotides 98–708 and 467–1811 of PDK1 (Figure 1), were obtained from the I.M.A.G.E. consortium [41] and sequenced. The two sequences were joined together by a *Sca*I restriction enzyme digest of each EST clone, and the appropriate restriction fragments obtained from these digests were ligated to generate a plasmid containing a continuous PDK1 sequence from nucleotides 154–1670. This construct was used as a template for a PCR reaction to generate an amino-terminal epitope-tagged Myc–PDK1 (amino acid residues 52–556) construct. This was achieved using the 5′ primer GCGG**AGATCT**GCCACCATGGAGCAGAAGCTGATCTCTGAAGA GGACTTGGACGGCACTGCAGCCGAGCCTCGG and the 3′ primer GCGG***GGTACC***TCACTGCACAGCGGCGTCCGGGTG, which incorporate a *Bgl*II site (bold) and a *Kpn*I site (bold italics). The resulting PCR fragment was subcloned into the *Bgl*II–*Kpn*I sites of an pSP72 cloning vector, and the nucleotide sequence confirmed by DNA sequencing. The Myc–PDK1 coding sequence was subcloned from this vector as a *Bgl*II–*Kpn*I fragment into the *Bam*HI and *Kpn*I site of the eukaryotic expression vector pEBG-2T [42] in order to generate a construct for the expression of GST–PDK1 in 293 cells, while a *Bgl*II–*Sal*I fragment was ligated into pCMV5 [43] to generate a construct for the expression of Myc–PDK1 in 293 cells.

Amino-terminal Myc epitope tagged PDK1 constructs encoding the full-length protein (residues 1–556) and lacking the PH domain (residues 1–450) were generated by a PCR approach using the full-length PDK1 cDNA isolated from the MCF7 library. This was achieved by using the the 5′ primer GCGGAGATCTGCCACCATGGAGCAGAAGCTGATCTCTGAAGAGGACTTGGCCAGGACCAC CAGCCAGCTGTATGACG (for both the full-length and ΔPH-PDK1 constructs) and the 3′ primers GCGGGGTACCTCACTGCACAGCGGCGTCCGGGTG (for full-length PDK1) and GCGGGGTACCTCAGTGCCAAGGGTTTCCGCCAGCCTGCTT (for the ΔPH-PDK1 construct). The resulting PCR fragments were cloned into the pCR 2.1-TOPO vector and subsequently subcloned into the pEGB-2T vector as a *Bgl*II–*Kpn*I fragment and pCMV5 as an *Eco*RI–*Kpn*I fragment. A full-length catalytically inactive PDK1 construct in which Asp223 was changed to Ala was created by the PCR-based megaprimer strategy [44] and then subcloned into pCMV5 and pEBG-2T as described above. The structure of each construct was verified by DNA sequencing, and plasmid DNA for transfection was purified using the Qiagen plasmid Mega kit according to the manufacturer's protocol.

A bacterial expression vector for GST–DSTPK61 was prepared by ligating an *Eco*RI–*Eco*RV fragment of the *Dstpk61* cDNA derived from pBluescriptSK into the *Eco*RI–*Sal*I site of the pGEX-3X expression vector.

Preparation of DNA expression constructs encoding GST–ΔPH-PKBα

A PCR-based strategy was used to prepare a GST–ΔPH-PKBα (encoding residues 118–479 of PKBα) construct, using as a template a full-length human PKBα construct that was isolated from a human skeletal muscle cDNA library and subcloned into the pBluescriptSK vector (M. Deak and D. Alessi, unpublished data). The GST–ΔPH-PKBα construct was obtained using the 5′ primer CGGGATCCATGGACTTCCGGTCGGGCTCA and the 3′ primer was the T7 oligonucleotide of pBluescriptSK vector. The resulting PCR fragment was cloned into pBluescriptSK as a *Bam*H1–*Kpn*I fragment and subsequently into pEBG-2T as a *Bam*H1–*Kpn*I fragment.

Expression of GST–PDK1, GST–ΔPH-PDK1, GST–D223A-PDK1, GST–ΔPH-PKBα and GST–DSTPK61

Twenty 10 cm diameter dishes of human embryonic kidney 293 cells were cultured and each dish transfected with 20 μg DNA encoding GST–PDK1 (residues 52–556), GST–PDK1 (residues 1–556), GST–ΔPH-PDK1, GST–D223A-PDK1 or GST–ΔPH-PKBα using a modified calcium phosphate method [20]. 24 h after transfection, the cells were serum starved for 16 h and each dish of cells lysed in 1 ml of ice-cold Buffer A. The 20 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 1 ml of 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 of Buffer B to ensure complete removal of all the Triton X-100, which interferes with the activation of GST–PKBα by PDK1 [21]. GST–PDK1α was eluted from the resin at ambient temperature with three 1 ml portions of Buffer B containing 20 mM glutathione pH 8.0 and 0.27 M sucrose. The combined eluates were divided into aliquots, snap frozen in liquid nitrogen and stored at −80°C. Between 0.5 and 2.0 mg of each GST fusion protein were obtained and were more than 90% homogeneous as judged by SDS–polyacrylamide gel electrophoresis.

E. coli transformed with an expression plasmid encoding GST–DSTPK61 were grown at 37°C in LB medium to an absorbance of 0.6 at 600 nm. Isopropyl-β-D-thiopyranoside was added to 30 μM and the bacteria were incubated for 16 h at 25°C before centrifuging for 10 min at 4000 × g. The bacteria were resuspended in 15 ml of Buffer A and lysed by sonication for 4 min on ice. The suspension was centrifuged for 30 min at 30,000 × g and the GST–DSTPK61 was purified by affinity chromatography on 1 ml of glutathione-Sepharose as described above for GST–PDK1. The GST–DSTPK61 derived from this (0.35 mg/ml) was divided into aliquots, snap frozen in liquid nitrogen and stored at −80°C.

Assay of GST–PDK1 and GST–DSTPK61 activity

GST–PDK1 and GST–DSTPK61 were diluted appropriately in Buffer B containing 1 mg/ml bovine serum albumin and assayed for their ability to activate and phosphorylate GST–PKBα [21]. One unit of PDK1 activity was defined as that amount required to increase the basal activity of GST–PKBα by 1 unit of activity in 1 min; 1 unit of GST–PKBα activity was that amount of enzyme required to catalyse the phosphorylation of 1 nmol of the peptide RPRAATF in 1 min in an assay containing 0.1 mM RPRAATF [45]. In order to ensure that the assay was linear with respect to time, the concentration of GST–PDK1 or GST–DSTPK61 was kept below 2 units/ml. At this concentration, the level of phosphorylation of PKBα was < 0.4 moles of phosphate per mole of protein in the 30 min assay.

Transfection of 293 cells with HA-tagged PKBα and Myc–PDK1

Human embryonic kidney 293 cells were cultured on 10 cm diameter dishes and transfected with 1 mg/ml pCMV5 DNA constructs encoding HA–PKBα or HA–PKBα plus Myc–PDK1 or HA–PKBα plus Myc–D223A-PDK1 [20]. After 24 h the cells were deprived of serum for a further 16 h and then stimulated for 10 min with either 100 ng/ml IGF1 or Buffer C. The cells were lysed in 1.0 ml of ice-cold Buffer A, the lysate centrifuged at 4°C for 10 min at 13,000 × g and HA–PKBα immunoprecipitated from aliquots of the supernatant (10 mg protein) [20] and assayed for PKBα activity with the peptide RPRAATF [45] as described previously [20].

^{32}P-labelling of 293 cells transfected with HA–PKBα or Myc–PDK1

This was carried out essentially as described previously [20]. Briefly, 293 cells were transfected with HA–PKBα, or with HA–PKBα plus Myc–PDK1, washed with phosphate-free Dulbecco's modified Eagle's medium (DMEM), incubated for 4 h with ^{32}P-orthophosphate (1 mCi/ml), then stimulated with IGF1 (100 ng/ml) for 5 min. The cells were lysed and the HA–PKBα immunoprecipitated (using 10 μg of 12CA5 antibody per 10 cm dish of cells) or the Myc–PDK1 immunoprecipitated (using 10 μg of 9E10 antibody per 10 cm dish of cells). The immunoprecipitated protein was alkylated with 4-vinylpyridine, subjected to SDS–polyacrylamide gel electrophoresis, digested with trypsin and analysed by chromatography on a C_{18} column [20].

Measurement of PDK1 activity in 293 cell extracts.
293 cells cultured on 10 cm diameter dishes (five dishes for each determination of PDK1 activity) were deprived of serum for a further 16 h and then stimulated for 2 or 10 min with either 100 ng/ml IGF1 or Buffer C. The cells were lysed in 1.0 ml of ice-cold Buffer A containing 0.2 M NaCl, the lysate centrifuged at 4°C for 10 min at 13,000 × g and applied to a 1 ml heparin-Sepharose column (HiTrap) equilibrated in Buffer B containing 0.2 M NaCl, and the column washed until the absorbance at 280 nm had decreased back to the baseline. The column was developed with a 40 ml linear salt gradient to 2.0 M NaCl and 1.0 ml fractions were collected. The column fractions were assayed for PDK1 activity in the presence and absence of PtdCho/PtdSer lipid vesicles containing PtdIns(3,4,5)P_3 [21].

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