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25. Whole-cell extracts were prepared by lysing the cells in NET-N buffer containing 50 mM tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM dithiothreitol, 0.1% NP-40, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of leupeptin, pepstatin, and aprotinin). The mixture was then centrifuged at 30,000*g* for 1 hour at 4°C, and the supernatant was stored at −80°C until use. For coimmunoprecipitation assays, 1 mg of cell extract was incubated in the presence of 2 μg of anti-p/CIP, anti-Flag, or anti-RAR IgG for 2 hours at 4°C. The immune complexes were then precipitated with protein A–Sepharose. Protein complexes were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) [U. K. Laemmli, *Nature* **227**, 680 (1970)], protein immunoblotted, and probed using 1 μg/ml of anti-Flag, anti-p/CIP, or an anti-hemagglutinin (anti-HA).
26. The yeast strain EGY 48, the LexA-b galactosidase reporter construct (PSH 18-34), and the B42 parental vectors (PEG 202 and PJG 4-5) were all previously described [J. Gyuris *et al.*, *Cell* **75**, 791 (1993)]. Various p/CAF fragments or fragments of other coactivators were obtained by polymerase chain reaction or restriction digestion and subcloned into PEG 202 bait vector or PJG 4-5 prey vectors, respectively. EGY 48 cells were transformed with the *lac Z* reporter plasmid pSH 18-34 with the appropriate bait and prey vectors, and were plated out on Ura$^-$His$^-$Trp$^-$ medium containing 2% galactose. Isolated yeast colonies were then allowed to grow in the same liquid medium and were subsequently assayed for β-galactosidase, as previously described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1995)]. All constructs were sequenced and functionally tested in yeast two-hybrid assays, showing interaction with other proteins as previously described (*2*, *7*). In the case of p/CAF constructs, each fragment interacted positively with at least one other coactivator except the fragment with amino acids 86 through 518.
27. Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass cover slips at subconfluent density and grown in D-MEM/F-12 (Life Technologies) medium supplemented with 10% fetal bovine serum, gentamicin, and methotrexate. Before the injection, the cells were rendered quiescent by incubation in serum-free medium for 24 to 36 hours. Plasmids were injected into the nuclei of cells at a final concentration of 100 μg/ml. Immunoglobulin G specific for p/CAF was prepared from guinea pig serum raised against a bacterially expressed fragment (amino acids 466 to 832) of p/CAF; this IgG recognized the single band of p/CAF in protein immunoblot analysis. Either preimmune IgG or the appropriate specific antibodies directed against p/CAF, p/CIP, SRC-1, or CBP were coinjected and allowed the unambiguous identification of the injected cells (*7*). Preimmune controls were included in all experiments. Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately 1 hour after injection, the cells were stimulated, where indicated, with the appropriate ligand. In the case of rescue experiments, the cells were stimulated with ligand 6 hours after injection to allow protein expression. After overnight incubation, the cells were fixed and then stained to detect injected IgG and β-galactosidase expression [D. W. Rose *et al.*, *J. Cell Biol.* **119**, 1405 (1992); (*2*)]. Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. All experimental results are expressed as the mean ± SEM of at least three experiments in which at least 1000 cells were injected.
28. GST-RAR and GST-CBP fragments were generated as described (*2*). We incubated 25 μl of GST-Sepharose beads containing 3 to 6 μg of the GST recombinant proteins with 5×10^5 cpm of ^{35}S-labeled p/CAF proteins generated by in vitro transcription and translation for 2 hours at 4°C. The complexes were washed five times with NET-N buffer, resolved by SDS-PAGE, and fluorographed.
29. Mutations in p/CAF and CBP were introduced by site-directed mutagenesis using the Quick-Change mutagenesis system (Stratagene) according to the manufacturer's instructions. Double-stranded oligonucleotides were designed such that the wild-type sequence corresponding to amino acids Tyr^{616}/Phe^{617} (acetyl-CoA–binding site) in p/CAF cDNA were substituted with alanines in order to generate a mutant of p/CAF lacking HAT activity (pCMV–p/CAF$_{HAT-}$). A similar strategy was used to obtain mutants of CBP. Mutants of p/CAF and CBP were expressed in bacteria and baculovirus, respectively, and tested for HAT activity in solution using histones as substrates [J. E. Brownell and C. D. Allis, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6364 (1995)].
30. We thank R. Heyman for use of TTNPB and LG629, S. L. Berger for discussion and providing hGCN5 expression vector, and Y. Nakatani and X. J. Yang for providing a tagged p/CAF expression vector. We also thank C. Nelson for experimental assistance, L.-M. Phillips for excellent technical assistance, and P. Myer for expertise in figure preparation. E.K. is supported by a U.S. Army Medical Research Program Award, J.T. by the National Cancer Institute of Canada, E.M.M. by an NIH Postdoctoral Fellowship, D.W.R. by an American Diabetes Association Career Development Award, and L.X. by an American Heart Association Predoctoral Fellowship. Supported by grants from NIH to C.K.G. and M.G.R.

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Phosphorylation and Activation of p70^{s6k} by PDK1

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Activation of the protein p70^{s6k} by mitogens leads to increased translation of a family of messenger RNAs that encode essential components of the protein synthetic apparatus. Activation of the kinase requires hierarchical phosphorylation at multiple sites, culminating in the phosphorylation of the threonine in position 229 (Thr^{229}), in the catalytic domain. The homologous site in protein kinase B (PKB), Thr^{308}, has been shown to be phosphorylated by the phosphoinositide-dependent protein kinase PDK1. A regulatory link between p70^{s6k} and PKB was demonstrated, as PDK1 was found to selectively phosphorylate p70^{s6k} at Thr^{229}. More importantly, PDK1 activated p70^{s6k} in vitro and in vivo, whereas the catalytically inactive PDK1 blocked insulin-induced activation of p70^{s6k}.

The p70 ribosomal protein S6 kinase, p70^{s6k}, participates in the translational control of mRNA transcripts that contain a polypyrimidine tract at their transcriptional start site (*1*). Although these transcripts represent only 100 to 200 genes, they can encode up to 20% of the cell's mRNA (*2*), with most of these transcripts encoding components of the translational apparatus (*2*). The p70^{s6k} has been tentatively identified as a downstream effector of the phosphoinositide 3-kinase (PI3K) signaling pathway, but the upstream kinases linking PI3K with p70^{s6k} have not been identified (*3*). In addition, p70^{s6k} activity is controlled by the mammalian target of rapamycin (mTOR), which appears to protect the kinase from a phosphatase (*4*). Activation of p70^{s6k} is associated with increased phosphorylation of eight residues (Fig. 1A) (*4*). Of these, three appear to be essential in regulating kinase activation, including Thr^{229} in the catalytic domain as well as Ser^{371} and Thr^{389} in the linker domain (Fig. 1A) (*4*, *5*). All three sites are conserved in many members of the AGC (protein kinases A, G, and C) family of Ser/Thr kinases, including PKB and PKC. The sequence surrounding Thr^{229} is also highly conserved in the Ca^{2+}- and calmodulin-dependent protein kinase family (CaMK), the closest neighbor to that of the AGC family (Fig. 1A) (*6*). Recently, it was demonstrated that a newly described kinase, termed PDK1, phosphorylates the equivalent site to Thr^{229} in PKB, Thr^{308} (*7*). Further studies have indicated that although PDK1 is constitutively active, its ability to phosphorylate Thr^{308} is blocked by the NH_2-terminal pleckstrin ho-

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mology (PH) domain of PKB (*7*). Upon mitogen stimulation, PI3K increases the level of phosphatidylinositide-3,4,5-P_3, which binds to the PH domain of PKB, presumably disrupting its interaction with Thr^{308} (*8*). The in vivo kinase that phosphorylates Thr^{229} in the catalytic domain of $p70^{s6k}$ is also thought to be constitutively active, but requires prior phosphorylation of Thr^{389}, in the linker region of $p70^{s6k}$ (Fig. 1A), to bring about kinase activation (*9*).

To investigate whether PDK1 phosphorylates Thr^{229} in $p70^{s6k}$, Myc-tagged PDK1 (*10*) and Myc-$p70^{s6k}$ were expressed in 293 cells. As a control, PDK1 activity was assessed with an influenza hemagglutinin-tagged PKB variant lacking the PH domain, termed HA-ΔPHPKB (*11*), which is a substrate for PDK1 in the absence of phospholipids (*8*). Myc-PDK1 phosphorylated HA-ΔPHPKB, whereas the catalytically inactive variant of PDK1, Myc-PDK1-KD, did not (Fig. 1B) (*12*). Incubation of Myc-PDK1 with Myc-$p70^{s6k}$ led to only a small increase in the amount of phosphate incorporated into Myc-$p70^{s6k}$ (Fig. 1C). The phosphorylation of Thr^{229} appears to depend on phosphorylation of Thr^{389} (*13*), and this reaction is facilitated by converting four (Ser/Thr)-Pro phosphorylation sites in the autoinhibitory domain to acidic residues (Fig. 1A) (*14*). Therefore, we tested whether a $p70^{s6k}$ variant with acidic residues placed at Thr^{389} and at the four (Ser/Thr)-Pro sites (Myc-$p70^{s6k}$E389D_3E) (*14*) would serve as a better substrate for PDK1. The Myc-$p70^{s6k}$E389D_3E protein was more efficiently phosphorylated by PDK1 than the wild-type enzyme or the Myc-$p70^{s6k}D_3$E variant, in which only the (Ser/Thr)-Pro sites are converted to acidic residues (Fig. 1C). Myc-PDK1-KD did not phosphorylate Myc-$p70^{s6k}$E389D_3E (Fig. 1C). To verify the site of phosphorylation, the Myc-$p70^{s6k}$E389D_3E variant and a mutant in which Thr^{229} was converted to serine (*15*) were phosphorylated in vitro by Myc-PDK1 and subjected to two-dimensional phosphopeptide mapping (*9*). In both cases, a single major phosphopeptide was observed (Fig. 1D) that migrated at the identical position. Amino acid analysis of the two phosphopeptides revealed phosphothreonine in the peptide from the Myc-$p70^{s6k}$E389D_3E mutant and phosphoserine in the peptide from the Ser^{229} mutant (Fig. 1D). Thus, PDK1 selectively phosphorylates $p70^{s6k}$ at position Thr^{229}.

Only the active form of PDK1 phosphorylates and activates HA-ΔPHPKB in vitro (Fig. 2A) (*8*). In the case of Myc-$p70^{s6k}$ and Myc-$p70^{s6k}D_3$E, the small increase in phosphorylation catalyzed by Myc-PDK1 had no detectable effect on activity (Fig. 2B). This finding is consistent with the inability of Myc-PDK1 to phosphorylate Thr^{389}, which appears to be an absolute requirement for $p70^{s6k}$ activation (*9*, *14*). Although Myc-$p70^{s6k}$E389D_3E has high basal kinase activity, Myc-PDK1 further increased the activity of this variant (Fig. 2B). The catalytically inactive Myc-PDK1-KD did not activate Myc-$p70^{s6k}$E389D_3E (Fig. 2B). These results support the hypothesis that phosphorylation of Thr^{389} is a prerequisite for phosphorylation of Thr^{229}.

The threonine residues in the catalytic domains of $p70^{s6k}$ and PKB appear to be selectively phosphorylated by PDK1. Members of CaMK family of protein kinases also have similar amino acid sequences in their catalytic domains (Fig. 1A) (*6*). The catalytic domain kinase for a member of the CaMK family, CaMK IV, has been cloned and termed CaMK kinase (*16*), allowing

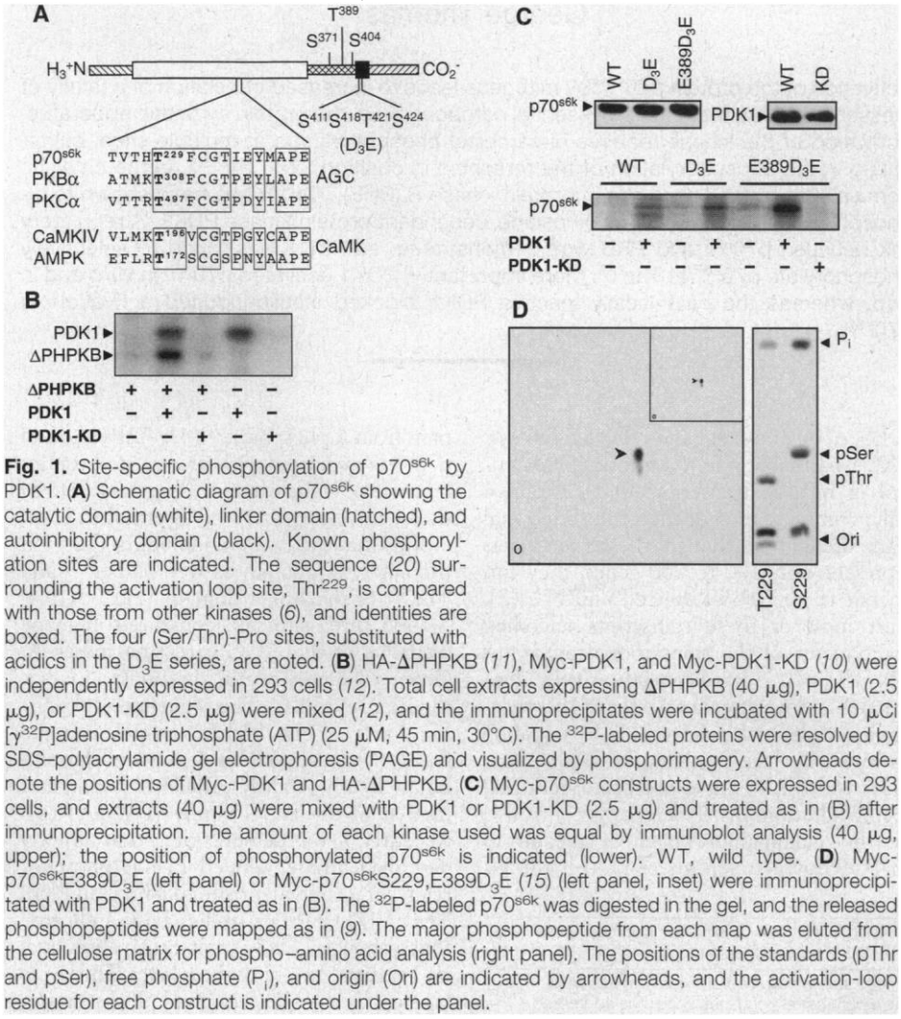

Fig. 1. Site-specific phosphorylation of $p70^{s6k}$ by PDK1. (**A**) Schematic diagram of $p70^{s6k}$ showing the catalytic domain (white), linker domain (hatched), and autoinhibitory domain (black). Known phosphorylation sites are indicated. The sequence (*20*) surrounding the activation loop site, Thr^{229}, is compared with those from other kinases (*6*), and identities are boxed. The four (Ser/Thr)-Pro sites, substituted with acidics in the D_3E series, are noted. (**B**) HA-ΔPHPKB (*11*), Myc-PDK1, and Myc-PDK1-KD (*10*) were independently expressed in 293 cells (*12*). Total cell extracts expressing ΔPHPKB (40 μg), PDK1 (2.5 μg), or PDK1-KD (2.5 μg) were mixed (*12*), and the immunoprecipitates were incubated with 10 μCi [γ^{32}P]adenosine triphosphate (ATP) (25 μM, 45 min, 30°C). The ^{32}P-labeled proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by phosphorimagery. Arrowheads denote the positions of Myc-PDK1 and HA-ΔPHPKB. (**C**) Myc-$p70^{s6k}$ constructs were expressed in 293 cells, and extracts (40 μg) were mixed with PDK1 or PDK1-KD (2.5 μg) and treated as in (B) after immunoprecipitation. The amount of each kinase used was equal by immunoblot analysis (40 μg, upper); the position of phosphorylated $p70^{s6k}$ is indicated (lower). WT, wild type. (**D**) Myc-$p70^{s6k}$E389D_3E (left panel) or Myc-$p70^{s6k}$S229,E389D_3E (*15*) (left panel, inset) were immunoprecipitated with PDK1 and treated as in (B). The ^{32}P-labeled $p70^{s6k}$ was digested in the gel, and the released phosphopeptides were mapped as in (*9*). The major phosphopeptide from each map was eluted from the cellulose matrix for phospho–amino acid analysis (right panel). The positions of the standards (pThr and pSer), free phosphate (P_i), and origin (Ori) are indicated by arrowheads, and the activation-loop residue for each construct is indicated under the panel.

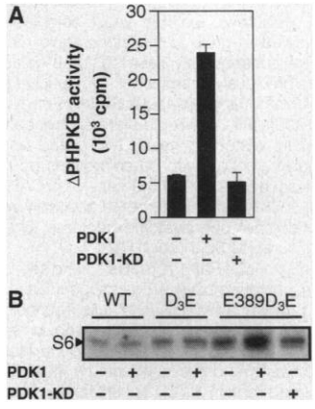

Fig. 2. Activation of $p70^{s6k}$ and PKB by PDK1 in vitro. Equal amounts of either (**A**) HA-ΔPHPKB or (**B**) Myc-tagged variants of $p70^{s6k}$ were immunoprecipitated with either Myc-PDK1 or Myc–PDK1-KD as in Fig. 1B. The ΔPHPKB and $p70^{s6k}$ were activated by PDK1 by incubation with 200 μM MgATP. The PKB activity was measured with Crosstide (*19*) by filter paper assay (A), and $p70^{s6k}$ activity, with 40S ribosomal subunits (*9*) (B). Phosphorylated S6 was resolved by SDS-PAGE and visualized by phosphorimagery. Each $p70^{s6k}$ variant used and phosphorylated S6 are indicated. The results are representative of two independent experiments.

the possibility of testing the two kinases against alternative substrates. Purified Myc-PDK1 did not phosphorylate CaMK IV (Fig. 3A). Similarly, CaMK kinase did not appreciably phosphorylate either purified glutathione S-transferase (GST)–tagged p70^{s6k}E389D$_3$E or HA-ΔPHPKB (Fig. 3A). Thus, the upstream kinases for the catalytic domains of the AGC and CaMK protein kinase families are apparently selective for their respective family members.

Earlier studies indicated that the Thr^{229} kinase is constitutively active in quiescent cells and resistant to wortmannin and rapamycin, agents known to block p70^{s6k} phosphorylation and activation (9). Consistent with this result, the activity of PDK1 has been reported to be constitutive (8). We therefore immunoprecipitated Myc-PDK1 from quiescent or insulin-stimulated 293 cells treated with wortmannin or rapamycin. Myc-PDK1 was then tested for its ability to phosphorylate Myc-p70^{s6k}E389D$_3$E in vitro. The activity of PDK1 was unaffected by insulin stimulation, and treatment of cells with either wortmannin or rapamycin did not inhibit PDK1 activity (Fig. 3B). Thus, PDK1 is constitutively active and insensitive to agents known to block insulin-induced p70^{s6k} activation.

The observations above suggest that PDK1 is the kinase responsible for regulating p70^{s6k}E389D$_3$E activation in vivo. Expression of Myc-PDK1 slightly enhanced basal activity of a Myc-p70^{s6k}-GST reporter, but potently activated Myc-p70^{s6k}E389D$_3$E-GST, an effect that was not further augmented by insulin (Fig. 4A). In contrast, expression of catalytically inactive Myc-PDK1-KD blocked insulin-induced activation of Myc-p70^{s6k}E389D$_3$E-GST (Fig. 4A) as well as Myc-p70^{s6k}-GST (*13*). The specificity of PDK1 was further supported by the finding that co-expression of PDK1 with the mitogen-activated protein kinase (p44^{mapk}) had no effect on either basal or 12-O-tetradecanoylphorbol 13-acetate (TPA)–induced p44^{mapk} (Fig. 4B). In addition, Myc-PDK1 activation of Myc-p70^{s6k}E389D$_3$E was not inhibited by wortmannin or rapamycin (Fig. 4C). Co-expression of Myc-PDK1 also increased HA-PKB activity; however, the effect on ΔPHPKB activity was much larger (Fig. 4D). Thus, PDK1 can increase the activity of p70^{s6k} and PKB in vivo, if the regulatory constraints that would normally block access to the catalytic domain phosphorylation site are removed.

The results presented here are consistent

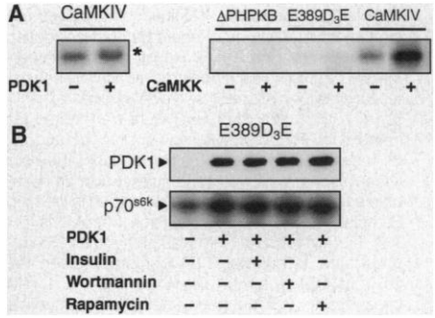

Fig. 3. Specificity of catalytic domain kinases. (**A**) After immunoprecipitation, HA-ΔPHPKB and Myc-PDK1 were eluted from immune complexes with HA and Myc epitope peptides, respectively, in buffer B. Myc-p70^{s6k}E389D$_3$E-GST, from transfected 293 cells, was purified on glutathione-Sepharose and then eluted with 10 mM glutathione in buffer B (*12*). The purity and concentration of each kinase were verified by SDS-PAGE and Coomassie staining. Equal amounts (200 ng) of purified, soluble HA-ΔPHPKB, Myc-p70^{s6k}E389D$_3$E-GST, or CaMK IV were incubated with soluble Myc-PDK1 or CaMK kinase (*18*) and Mg[γ-^{32}P]ATP as in Fig. 1B. Phosphorylated products were resolved by SDS-PAGE and visualized by phosphorimagery. Each substrate is indicated above and the phosphorylating kinase is indicated below the panel. The asterisk indicates the position of autophosphorylated Myc-PDK1. The results are representative of two independent experiments. (**B**) Myc-PDK1 was expressed in 293 cells and then either extracted immediately, stimulated with insulin (1 μM, 10 min) and extracted, or treated (30 min) with wortmannin (250 nM) or rapamycin (20 nM) and extracted. After immunoprecipitation, Myc-PDK1 (expression, top panel) activity was measured with purified Myc-p70^{s6k}E389D$_3$E-GST (200 ng) as in (A). The ^{32}P-labeled Myc-p70^{s6k}E389D$_3$E-GST was visualized after SDS-PAGE and phosphorimagery (bottom panel). The results are representative of two independent experiments.

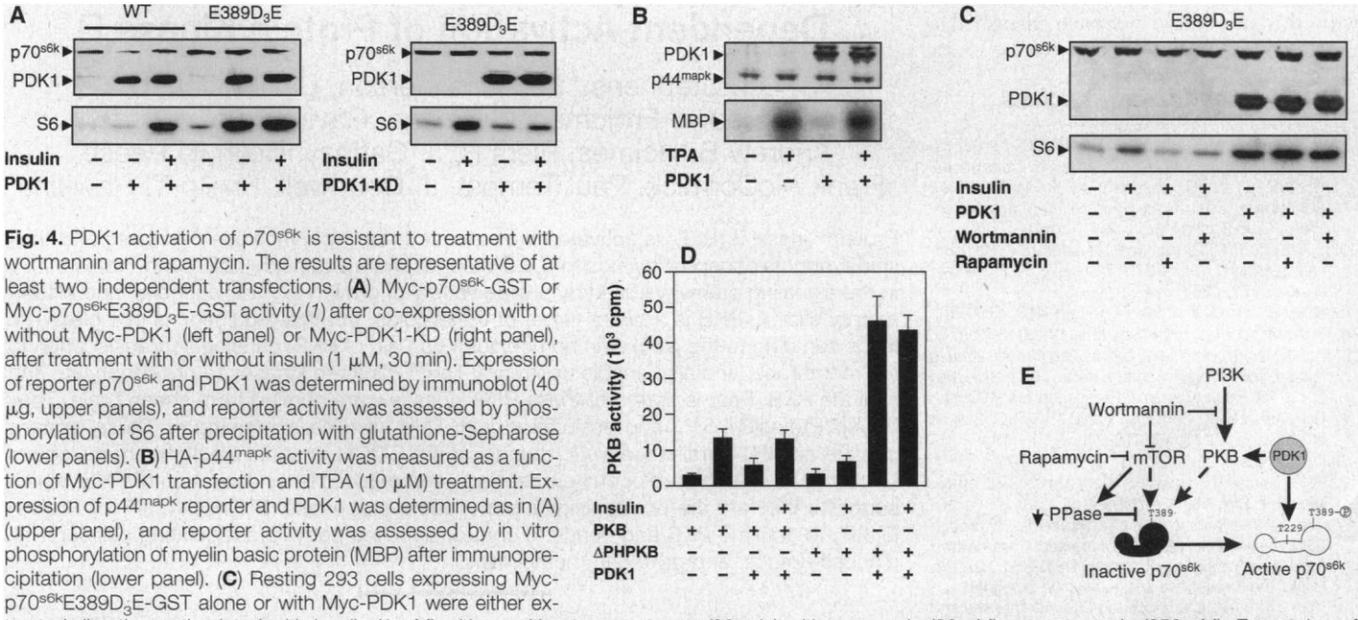

Fig. 4. PDK1 activation of p70^{s6k} is resistant to treatment with wortmannin and rapamycin. The results are representative of at least two independent transfections. (**A**) Myc-p70^{s6k}-GST or Myc-p70^{s6k}E389D$_3$E-GST activity (*1*) after co-expression with or without Myc-PDK1 (left panel) or Myc-PDK1-KD (right panel), after treatment with or without insulin (1 μM, 30 min). Expression of reporter p70^{s6k} and PDK1 was determined by immunoblot (40 μg, upper panels), and reporter activity was assessed by phosphorylation of S6 after precipitation with glutathione-Sepharose (lower panels). (**B**) HA-p44^{mapk} activity was measured as a function of Myc-PDK1 transfection and TPA (10 μM) treatment. Expression of p44^{mapk} reporter and PDK1 was determined as in (A) (upper panel), and reporter activity was assessed by in vitro phosphorylation of myelin basic protein (MBP) after immunoprecipitation (lower panel). (**C**) Resting 293 cells expressing Myc-p70^{s6k}E389D$_3$E-GST alone or with Myc-PDK1 were either extracted directly or stimulated with insulin (1 μM) with or without pretreatment (30 min) with rapamycin (20 nM) or wortmannin (250 nM). Expression of Myc-p70^{s6k}E389D$_3$E-GST and Myc-PDK1 was determined (upper panel), and reporter p70^{s6k} activity was assessed by S6 phosphorylation (lower panel) as in (A). (**D**) The effect of insulin on HA-PKB and HA-ΔPHPKB activity, with or without PDK1 co-expression, was measured with Crosstide (*19*); expression of each construct was observed to be equal by immunoblot analysis (data not shown). (**E**) A model of p70^{s6k} activation predicts Thr^{229} phosphorylation by PDK1 after prior phosphorylation of Thr^{389}. Tentative placement of other potential components in the activation scheme are indicated with a dotted line. PPase, phosphatase.

with PDK1 as the in vivo kinase responsible for mediating Thr^{229} phosphorylation in the catalytic domain of $p70^{s6k}$. The kinases responsible for mediating activation of $p70^{s6k}$ have been difficult to identify because of the multiple and hierarchical regulatory steps required to bring about its activation (*4*, *9*, *14*). Initially, it had been suggested that Thr^{229} phosphorylation was regulated by a kinase that was activated directly or indirectly by PI3K in a wortmannin-sensitive manner (*17*). However, our studies indicate that the Thr^{229} kinase is constitutively active, wortmannin-resistant (*9*), and dependent on prior phosphorylation of Thr^{389} to provide access to Thr^{229} (*13*). These latter requirements are fulfilled by PDK1 (Fig. 4E) (*4*, *9*). Activation of $p70^{s6k}$ appears to be first mediated by phosphorylation of the (Ser/Thr)-Pro sites in the autoinhibitory domain, which facilitates phosphorylation at Thr^{389} by disrupting the interaction of the COOH- and NH_2-termini of the kinase, thereby allowing phosphorylation of Thr^{229} (Fig. 4E) (*4*, *9*). A key step in this process is Thr^{389} phosphorylation, which appears to be positively regulated by a wortmannin-sensitive, PI3K-dependent input, possibly through PKB, and is suppressed by a rapamycin-activated Thr^{389} phosphatase (*9*), through inhibition of mTOR (Fig. 4E). Many members of the AGC family of Ser/Thr kinases share the same conserved catalytic domain of $p70^{s6k}$ and PKB (Fig. 1A) (*6*), suggesting that PDK1 may be a member of a family of kinases that mediate activation-loop phosphorylation of AGC protein kinases. Consistent with this possibility, we have identified a number of PDK1-like cDNAs (*18*).

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10. The cDNA encoding PDK1 (amino acids 52 through 556) was isolated with use of the sequence derived from the *Drosophila* homolog, DSTPK61/Y07908. Using the *Drosophila* sequence, we screened human expressed sequence tag (EST) databases and assembled an open reading frame covering amino acids 52 through 556. This open reading frame was confirmed by screening the Institute for Genomic Research (TIGR) EST database. The cDNA was obtained by polymerase chain reaction (PCR) from a placenta library (Clonetech) with use of the following oligonucleotides: 5′-CGC ATG GAC GGC ACT GCA GCC GAG CCT CG and 3′-CGC AGG CCA CGT CAC TGC ACA GCG GCG. After confirmation of the sequence, a Myc epitope (EQKLISEEDL) was added by a second round of PCR (5′-CCC GGT ACC GCC ATG GAA CAG AAA CTC ATC TCT GAA GAG GAT CTG GAC GGC ACT GCA GCC GAG CCT CG and 3′-CCC TCT AGA TCA CTG CAC AGC GGC GTC CGG GTG GC). The cDNA was subcloned as a Kpn I–Xba I fragment into pCMV5 and used in subsequent experiments. We generated Myc-tagged PDK1-KD (PDK1Q61) using Quickchange (Stratagene) with 5′-CGC TTC TCC AGA ATC TGA ATC GCA TAT TCT CTG G and 3′-CCA GAG AAT ATG CGA TTC AGA TTC TGG AGA AGC G oligonucleotides on the pCMV5-PDK1 construct.
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12. The 293 cells were seeded at 10^6 per 10-cm plate in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) 24 hours before transfection as previously described (*9*). Transfections were performed by titrating each construct to achieve consistent and uniform expression, typically no more than 1 μg of $p70^{s6k}$, PDK1, or PKB were transfected together with pRK5 or pCMV5 carrier DNA to 5 μg total (*9*). After calcium phosphate transfection, the cells were washed with DMEM and quiesced for 24 hours before extraction or treatments described in the text. Samples were extracted in buffer A [50 mM tris (pH 7.5), 50 mM NaCl, 10 mM NaF, 10 mM β-glycerol-phosphate, 10 mM $NaPP_i$ (P_i, inorganic phosphate), 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% TX-100, and 10 μg of leupeptin and aprotinin per milliliter]. We co-immunoprecipitated Myc-PDK1 and Myc-$p70^{s6k}$ or HA-ΔPHPKB by mixing extract from independently transfected cells in buffer A with the appropriate monoclonal antibody for each epitope tag. After immunoprecipitation with protein G–Sepharose, the samples were washed twice with buffer A, twice with buffer A containing 500 mM NaCl, and finally with buffer B [50 mM tris (pH 7.5), 10 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM benzamidine, and 0.2 mM PMSF]. The washed immunoprecipitates were resuspended in buffer B containing 10 mM $MgCl_2$ for assays described. The $p70^{s6k}$, PKB, and $p44^{mapk}$ activities were assayed as described in (*1*, *19*).
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Protein Kinase B Kinases That Mediate Phosphatidylinositol 3,4,5-Trisphosphate–Dependent Activation of Protein Kinase B

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Protein kinase B (PKB) is activated in response to phosphoinositide 3-kinases and their lipid products phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] and PtdIns(3,4)P_2 in the signaling pathways used by a wide variety of growth factors, antigens, and inflammatory stimuli. PKB is a direct target of these lipids, but this regulation is complex. The lipids can bind to the pleckstrin homologous domain of PKB, causing its translocation to the membrane, and also enable upstream, Thr^{308}-directed kinases to phosphorylate and activate PKB. Four isoforms of these PKB kinases were purified from sheep brain. They bound PtdIns(3,4,5)P_3 and associated with lipid vesicles containing it. These kinases contain an NH_2-terminal catalytic domain and a COOH-terminal pleckstrin homologous domain, and their heterologous expression augments receptor activation of PKB, which suggests they are the primary signal transducers that enable PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 to activate PKB and hence to control signaling pathways regulating cell survival, glucose uptake, and glycogen metabolism.

Phosphoinositide 3-kinases (PI3Ks) are a diverse family of enzymes capable of 3-phosphorylating inositol phospholipids (*1*). One subfamily can be activated by receptors through various signal transduction mechanisms. These enzymes phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 in vitro and apparently preferentially phosphorylate the