A 3-Phosphoinositide-dependent Protein Kinase-1 (PDK1) Docking Site Is Required for the Phosphorylation of Protein Kinase Cζ (PKCζ) and PKC-related Kinase 2 by PDK1

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Members of the AGC subfamily of protein kinases including protein kinase B, p70 S6 kinase, and protein kinase C (PKC) isoforms are activated and/or stabilized by phosphorylation of two residues, one that resides in the T-loop of the kinase domain and the other that is located C-terminal to the kinase domain in a region known as the hydrophobic motif. Atypical PKC isoforms, such as PKCζ and the PKC-related kinases, like PRK2, are also activated by phosphorylation of their T-loop site but, instead of possessing a phosphorylatable Ser/Thr in their hydrophobic motif, contain an acidic residue. The 3-phosphoinositide-dependent protein kinase (PDK1) activates many members of the AGC subfamily of kinases in vitro, including PKCζ and PRK2 by phosphorylating the T-loop residue. In the present study we demonstrate that the hydrophobic motifs of PKCζ and PKCζ, as well as PRK1 and PRK2, interact with the kinase domain of PDK1. Mutation of the conserved residues of the hydrophobic motif of full-length PKCζ, full-length PRK2, or PRK2 lacking its N-terminal regulatory domain abolishes or significantly reduces the ability of these kinases to interact with PDK1 and to become phosphorylated at their T-loop sites in vivo. Furthermore, overexpression of the hydrophobic motif of PRK2 in cells prevents the T-loop phosphorylation and thus inhibits the activation of PRK2 and PKCζ. These findings indicate that the hydrophobic motif of PRK2 and PKCζ acts as a “docking site” enabling the recruitment of PDK1 to these substrates. This is essential for their phosphorylation by PDK1 in cells.

Stimulation of cells with growth factors, phorbol esters, and insulin induces the activation of certain members of the AGC subfamily of protein kinases that include protein kinase B (PKB)1 (1, 2), p70 S6 kinase (p70 S6K) (3, 4), serum and glucocorticoid-induced kinase (SGK), (5–7) and many protein kinase C (PKC) isoforms (8, 9). These kinases mediate many of the cellular effects of agonists that elicit their activation by phosphorylating key regulatory proteins.

Recent work has led to a greater understanding of how these protein kinases are activated in cells. For example, PKB is turned on following the agonist-induced activation of PI 3-kinase which generates the second messenger PtdIns(3,4,5)P3, leading to the recruitment of PKB to the plasma membrane where it becomes activated by phosphorylation of two residues, namely Thr-308 and Ser-473 (2, 10). Thr-308 lies in the T-loop of the kinase domain, and Ser-473 is located C-terminal to the catalytic domain, in a region termed the “hydrophobic motif.” Conventional and novel PKC isoforms (8), p70 S6K (11), p90 ribosomal S6 kinase (12), and SGK (5–7) also possess residues lying in equivalent sequence motifs to Thr-308 and Ser-473 of PKB, whose phosphorylation is required for activation and/or stabilization of these kinases in vivo. The residue equivalent to Thr-308 of PKB in the T-loop of the kinase domain lies in a sequence motif comprising Thr-Phe-Cys-Gly-Thr where the underlined Thr residue becomes phosphorylated in response to agonist stimulation of cells. The residue equivalent to Ser-473 of PKB in the hydrophobic motif lies in a consensus sequence Phe-Xaa-Xaa-Phe/Tyr, where Xaa can be any amino acid.

Like other members of the AGC subfamily, the atypical PKC isoforms (PKCζ, PKCζ, and PKCζ) as well as the related PKC isoforms (PKR1 and PKR2) possess a Thr residue lying in a sequence motif identical to Thr-308 of PKB whose phosphorylation is essential for the activation of these kinases (Thr-410 in PKCζ (13, 14), Thr-774 in PKR1, and Thr-816 in PKR2 (15, 16)). These kinases also possess a hydrophobic motif in which the aromatic residues are conserved, but where the phosphorylatable Ser/Thr is replaced by an acidic residue (Glu-579 in PKCζ). The protein kinase termed 3-phosphoinositide-dependent protein kinase-1 (PDK1) plays a central role in activating AGC subfamily members (reviewed in Refs. 17–19). PDK1 phosphorylates the T-loop residue of PKB (20–23), p70 S6K (11, 24), p90RSK (25, 26), SGK (5–7) conventional and novel PKC isoforms (14, 27), atypical PKC isoforms (13, 14, 28), and related PKC isoforms (15, 16).

The kinase domain of PDK1 interacts with a region of PRK2 encompassing its hydrophobic motif termed the PDK1-interacting fragment (PIF) (29). Mutation of the conserved aromatic residues in the PRK2 hydrophobic motif or mutation of the Asp residue to either Ala or Ser greatly weakens the interaction of PIF with PDK1, indicating that PIF binds to PDK1 via these residues (29). Interestingly, PIF prevents PDK1 from phosphorylatable Ser/Thr to PDK1 via these residues (29).

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Yeast Two-hybrid Analysis—The wild type PKD1 and the L155D/L155S-PDK1 mutants subcloned into pAS2-1 vector and the pACT2-PIF vector which encodes the C-terminal 26 amino acids of PRK2 have been described previously (31). Y190 strain yeasts were co-transformed with the indicated combinations of vectors and grown on 1% (w/v) agar plates lacking histidine at 30 °C until appearance of colonies. Yeast colonies were patched onto fresh agar, incubated overnight at 30 °C, and filter lifts taken. Reporter β-galactosidase activity of the transformants was tested by incubating filters in 5-bromo-4-chloro-3-indolyl β-n-galactosidase (X-Gal) at 30 °C for 4 h.

Cloning of PKD1—A PCR-based strategy was used to prepare a N-terminal flag epitope-tagged cDNA construct encoding residues 501–984 of the human PRK2 (ΔNT-PRK2) using as a template an EST encoding for the full-length PRK2. The ΔNT-PRK2 construct was obtained using the 5′ primer cgggatccgc-ccactacagatatggtagcataggtcgaacggagggagga and the 3′ primer, ggagctctggaagagttttttggtgctttgagaaaaattttcttttgagttgctttccttacta. This incorporates a 5′ BamHI site followed by an N-terminal FLAG epitope tag and a 3′ SacI site. The resulting PCR fragment was cloned into pCR-Topo 2.1 (Invitrogen) and subsequently subcloned as an EcoRI/EcoRI fragment into the pCMV5 vector (36) (to encode expression of FLAG ΔNT-PRK2) and as a BamHI-KpnI fragment into the pEBG-2T vector (37) (to encode for expression of GST-ΔNT-PRK2).

The full-length cDNA encoding N-terminal FLAG epitope-tagged PKD1 (residues 1–984) was expressed in two stages. First, an antibody recognizing the N-terminus of PKD1 was raised in sheep against the whole N-terminal PDK1 protein (29). Monoclonal antibodies coupled to the non-phosphorylated peptide and then passed through a column of CH-Sepharose as a template an EST encoding for this region of PRK2 (NCBI accession number AA101793, IMAGE number 550355) obtained from the IMAGE consortium (35). The NT-PRK2 construct was obtained using as a template an EST encoding for the N-terminal region of PRK2 (NCBI accession number AA480660, IMAGE number 882160) obtained from the IMAGE consortium (35) was the template with the 5′ primer, ggagctctggaagagttttttggtgctttgagaaaaattttcttttgagttgctttccttacta. This incorporates a 5′ BamHI site followed by an N-terminal FLAG epitope tag and a 3′ SacI site. The resulting PCR fragment was cloned into pCR-Topo 2.1 (Invitrogen). In the second stage a triple ligation was set up in which the full-length PRK2 was generated by subcloning into the EcoRI-KpnI sites of the pCMV5 vector (36) the N-terminal EcoRI-SacI fragment, together with the C-terminal SacI-KpnI fragment of PRK2. The resulting full-length PRK2 fragment was then subcloned into the pEBG2T (37) as a BamHI-KpnI fragment to produce a plasmid encoding for the expression of full-length GST-PRK2.

Cloning of PKCζ—A PCR-based strategy was used to prepare an N-terminal flag epitope-tagged cDNA construct encoding full-length mouse PKCζ using as the template a full-length cDNA clone, kindly provided by Walter Kolch (Beatson Institute, Glasgow, UK), and the 5′ primer actagttcatggcctcacacggactcctc. This incorporated an N-terminal FLG and SpeI restriction sites at both ends of the cDNA. The resulting PCR fragment was cloned into the pCR-Topo 2.1 vector (Invitrogen) and subsequently as a SpeI-SpeI fragment into both the pCMV5 vector (36) (for expression of FLAG-PKCζ) and the pEBG-2T vector (37) (for expression of GST-PKCζ).

The constructs encoding wild type PKD1 (21), the Leu-155 mutants of PKD1 (31), GST-PIF, the mutant GST-F97T-PIF (29), and GST (empty pEBG2T vector) have been described previously. The constructs used to express GST-PRK1 (827–942), GST-PRK2-(494–587), and GST-PKCζ(518–585), in Fig. 1C, were derived from PKD1-interacting clones obtained from a human brain library (see above) and subcloned into the pEBG2T. The construct used to express GST-PRK2 (914–984) in Fig. 6 was subcloned into pEBG2T as described previously (29).

Binding of PKCζ and PRK2 to Myc-PDK1—For the data presented in Figs. 2 and 3, 293 cells were cotransfected with 10 µg of the wild type or mutant PKD1 plasmid and 10 µg of either the wild type or mutant PKCζ or PRK2. 3 h post-transfection the cells were lysed in 0.6 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 mM sucrose, 1 mM microcystin-LR, 0.1% (by volume) β-mercaptoethanol, and 1 tablet of protease inhibitor mixture per 50 ml of buffer). The lysates were cleared by centrifugation at 13,000 × g for 10 min at 2 °C, and 0.5 ml of supernatant was incubated for 2 h at 4 °C with 50 µl of glutathione-Sepharose. The beads were washed twice in lysis buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and two further washes in lysis buffer. The beads were resuspended in 30 µl of buffer containing 100 mM Tris/HCl, pH 6.8, 4% (by mass) SDS, 20% (by volume) glycerol, and 200 mM dithiothreitol and subjected to SDS-polyacrylamide gel electrophoresis. The gels were either stained with Coomassie Blue or analyzed by immunoblotting with either anti-FLAG or anti-Myc antibodies (described below).

Materials—The peptide used to assay PRK2 (PRKtide, AKRRRLSS-LRA) (32) and the peptides used to raise and affinity-purify the phospophosphorytic antibodies that recognize PRK2 phosphorylated at Thr-816 and PKCζ phosphorylated at Thr-410 were synthesized by Dr. G. Blomberg (University of Bristol, UK). The “Selectide” peptide used to assay PKCζ (AAKIGASFRGHMAKK) (33) was from Calbiochem. Protein G-Sepharose, glutathione-Sepharose, and activated-Sepharose were purchased from Amersham Pharmacia Biotech; protease-inhibitor mixture tablets were from Roche Molecular Biochemicals; tissue culture reagents and microcystin-LR were from Life Technologies, Inc., and secondary antibodies coupled to horseradish peroxidase were from Pierce.

Antibodies—The phospho-specific antibody recognizing PKR2 phosphorylated at Thr-816 and PKCζ phosphorylated at Thr-410 were synthesized by Dr. G. Blomberg (University of Bristol, UK). The “Selectide” peptide used to assay PKCζ (AAKIGASFRGHMAKK) (33) was from Calbiochem. Protein G-Sepharose, glutathione-Sepharose, and activated-Sepharose were purchased from Amersham Pharmacia Biotech; protease-inhibitor mixture tablets were from Roche Molecular Biochemicals; tissue culture reagents and microcystin-LR were from Life Technologies, Inc., and secondary antibodies coupled to horseradish peroxidase were from Pierce.

General Methods—Molecular biology techniques were performed using standard protocols. Site-directed mutagenesis was carried out using QuickChange kit (Stratagene) following instructions provided by the manufacturer. DNA constructs used for transfection were purified from bacteria using the Qiagen plasmid Mega kit according to the manufacturer’s protocol, and their sequence was verified using an automated DNA sequencer (model 373, Applied Biosystems). Human embryonic kidney 293 cells were cultured on 10-cm diameter dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and transfections were carried out using a modified calcium phosphate method (34). PD1K assays described in Fig. 1C were carried out as described previously (29).

Yeast Two-hybrid Screen—Myc-tagged human PKD1 was subcloned into the EcoRI/SalI site of pAS2-1 (CLONTECH) as a Gal4 DNA binding domain fusion. A yeast two-hybrid screen was carried out by transforming pAS2-1 PD1K and a pACT2 human brain cDNA library fused to the Gal4 activation domain (GAD) into the yeast strain Y190. The brain library was purchased from CLONTECH. Transformed yeast cells were incubated for 10 days at 30 °C on SD media supplemented with 25 mM 3-aminoantizole and lacking histidine, leucine, and tryptophan. Approximately 5 × 106 colonies were screened.
Activity and T-loop Phosphorylation of PKCζ and PRK2—For experiments shown in Fig. 4 and Fig. 5, 10 μg of either wild type PKCζ, mutant PKCζ, wild type PRK2, or mutant PRK2 plasmid was used. In Fig. 6, 2 μg of DNA construct encoding either PRK2 or PKCζ was cotransfected with 10 μg of DNA construct encoding either GST-F1P, GST-F977A-F1P, or GST. 36 h post-transfection the cells were lysed in 1 ml of lysis buffer. The lysates were centrifuged at 13,000 × g for 10 min at 2 °C, and the protein concentrations of the supernatant were determined by the Bradford method. To immunoprecipitate FLAG epitope-tagged PKCζ or PRK2, 50 μg of cell lysate protein was incubated with 2 μg of the FLAG antibody conjugated to 5 μl of protein A-Sepharose previously equilibrated in lysis buffer on a platform shaker for 60 min at 2 °C. The beads were then washed twice with 1 ml of lysis buffer containing 0.5 mM NaCl and twice with 1 ml of Buffer A (50 mM Tris/HCl, 0.1 mM EGTA, 0.27 M sucrose, and 0.1% (by volume) 2-mercaptoethanol), and the suspension was made up to a volume of 20 μl in Buffer A containing 25 mM glutathione. A mixture of other assay ingredients (30 μl) was then added to initiate the assay. The concentrations of reagents were 50 mM Tris, pH 7.5, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM EGTA, 10 μM PKA inhibitor (PKI), (29-32)ADFIASGRGTRNAHIDM, 100 μM [γ-32p]ATP (specific activity of ~500,000 cpm/nmol), 10 mM MgAc containing either 50 μM PKCζ peptide substrate (AAKIGASFRGHHMAK) or 30 μM PRK2 substrate peptide (AKRRRALSSLRA). After 10 min at 30 °C on a platform shaker, the reactions were terminated by pipetting 40 μl of the assay mixture onto 2 × 2-cm squares of phosphocellulose paper. These were washed in 75 mM phosphoric acid, and the amount of 32P-labeled peptide bound to the papers was determined as described previously for the assay of mitogen-activated protein kinase (38). One unit of activity was that amount of kinase that catalyzed the phosphorylation of 1 nmol of substrate in 1 min.

Immunoblotting—For the Myc and FLAG blots of cell lysate, 5 μg of protein was used. For the TS16-P blots, 25 μg of cell lysate protein was used. For the T410-P blots, 150 μg of cell lysate protein was immunoprecipitated using 5 μl of FLAG affinity gel and washed as described above. Cell lysates or immunoprecipitates were made 1% in SDS, subjected to SDS/polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The nitrocellulose membranes were immunoblotted using either the anti-Myc (0.4 μg/ml), anti-FLAG antibodies (0.4 μg/ml) in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5% (by volume) Tween (TBS/Tween), and 10% (by mass) skimmed milk. Immunoblotting with the phosphospecific antibodies (0.5 μg/ml) in the presence of 10 μg/ml phospho or dephospho peptide corresponding to the antigen used to raise the antibody in TBS/Tween containing 10% (by mass) skimmed milk. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

RESULTS

PKD1 Interacts with the Hydrophobic Motif of Atypical and Related PKC Isoforms—A yeast two-hybrid screen was carried out to identify proteins expressed in human brain that interact with PKD1. From this screen we identified a large number of positive clones that interacted with full-length PKD1 but not with the pleckstrin homology domain of PKD1, which corresponded to the C-terminal fragments of various PKC isoforms (Fig. 1A). These not only included PRK2 (3 positives), but also PKCζ (7 positives). Although each clone was of variable length, they all encompassed the C-terminal Phe-Xaa-Xaa-Phe-(Asp/Glu)-Phe/Tyr hydrophobic motif (Fig. 1B). The mutants L155E-

also obtained with two other protein preparations. 4 μg of each protein was electrophoresed on a 10% SDS-polyacrylamide gel and either immunoblotted using an antibody raised against the PKD1 protein to detect any endogenous PKD1 associated with the glutathione-Sepharose pull downs or stained with Coomassie Blue. The position of the molecular mass markers, ovalbumin (43 kDa) and carbonic anhydrase (29 kDa), are indicated. As reported previously in 293 cells, two PKD1-immunoreactive bands are observed running at 63 and 66 kDa (29).
PKD1, L155D-PKD1, and L155S-PKD1 which do not interact with the hydrophobic motif of PRK2 (31) were also unable to form a complex with the C-terminal fragments of PRK1, PKCζ, and PKCι (Fig. 1A).

In order to confirm by another procedure that the C-terminal regions of PRK1, PKCζ, PKCι, and PRK2 interacted with PKD1, these were expressed in mammalian 293 cells as glutathione S-transferase fusion proteins (see Fig. 1C). After their purification on glutathione-Sepharose, they were found to be associated with the endogenous PKD1 that was judged by Western blotting and by their ability to activate PKBα in the presence of MgATP and PtdIns(3,4,5)P3 (Fig. 1C). In contrast GST itself was not associated with any endogenous PKD1 when expressed in 293 cells and purified on glutathione-Sepharose or capable of inducing activation of PKBα (Fig. 1C). Taken together these results demonstrate that PKD1 directly interacts with the C-terminal regions of PRK1, PKCζ, PKCι, and PRK2 with significant affinity.

**PKD1 Interacts with the Hydrophobic Motif of PKCζ**—As reported previously (13, 14), a complex was readily observed between PKD1 and wild type PKCζ when these enzymes were coexpressed in 293 cells (Fig. 2A). In order to establish whether PKD1 was interacting with the hydrophobic motif of PKCζ, we mutated the conserved residues of the hydrophobic motif of PKCζ to Ala and tested whether these mutants were able to interact with wild type PKD1. Mutation of the aromatic residues in the hydrophobic motif of PKCζ greatly reduced its affinity for PKD1 (Fig. 2A). In contrast, mutation of Glu-579 to Ala, a residue that lies in the equivalent position to Ser-473 of PKBα, did not significantly affect the ability of PKD1 to bind PKCζ (Fig. 2A).

In order to establish whether PKCζ interacted with the PIF-binding pocket in the kinase domain of PKD1, we tested whether mutation of Leu-155 in the kinase domain of PKD1 affected the binding of PKCζ to PKD1. In Fig. 2B we demonstrate that mutation of Leu-155 to Ser, Asp, Glu, or Ala prevented the interaction of PKD1 with PKCζ, suggesting that the binding of PKCζ to PKD1 is mediated through the PIF-binding pocket on PKD1.

**PKD1 Also Interacts with the Hydrophobic Motif of PRK2**—Full-length PRK2 (Fig. 3A) and a PRK2 mutant that lacks the auto-inhibitory N-terminal domain (∆NT-PRK2, Fig. 3B) were capable of forming a stable interaction with wild type PKD1 when both enzymes were coexpressed in 293 cells, but they did not interact with PKD1 mutants in which Leu-155 was mutated to Ser, Asp, Glu, or Ala. Furthermore, mutation to Ala of the residue equivalent to Glu-579 of PKCζ in the hydrophobic motif of ∆NT-PRK2 (Asp-978) or the preceding conserved residue (Phe-977) also prevented the interaction of these enzymes with PKD1 (Fig. 3C).

**Disruption of the Hydrophobic Motif of PKCζ Inhibits Its Phosphorylation**—We prepared phospho-specific antibodies that only recognize PKCζ phosphorylated at Thr-410 (termed T410-P antibody), the site of PKD1 phosphorylation. These antibodies recognized wild type PKCζ expressed in 293 cells, and their specificity for Thr-410-phosphorylated PKCζ was established by the fact that recognition was abolished by preincubating the antibody with the phosphopeptide immunogen but not the dephospho form of this peptide (Fig. 4A). Furthermore, a mutant form of FLAG-PKCζ in which Thr-410 was changed to an Ala was not recognized by the T410-P antibody and, as reported previously (13), possessed virtually no activity (Fig. 4A). It should be noted that wild type PKCζ expressed in 293 cells was highly active when isolated from non-serum-starved cells. Furthermore, serum starvation of cells failed to reduce PKCζ activity, and stimulation of serum-starved cells with IGF1 under conditions that activate PI 3-kinase and PKBα did not induce any increase in PKCζ activity or phosphorylation at its T-loop. It therefore appears PKCζ expressed in 293 cells is constitutively phosphorylated at its T-loop residue.

In order to investigate the role of the hydrophobic motif of PKCζ in mediating phosphorylation of Thr-410, we tested the effect of mutating Phe-578, Glu-579, and Tyr-580 in the hydrophobic motif of PKCζ to Ala on the ability of PKCζ to become phosphorylated at its T-loop residue. The wild type and mutant forms of PKCζ were expressed in 293 cells to similar levels (Fig. 4B). The F578A-PKCζ mutant possessed 8% and Y580A-PKCζ mutants possessed 18% of the specific activity of the wild type PKCζ. Consistent with the inability of these mutants to interact with PKD1 (Fig. 2), they were barely phosphorylated at Thr-410 compared with the wild type kinase (Fig. 4B). In contrast...
contrast, the E579A-PKCζ mutant possessed significantly higher specific activity (55% of the wild type PKCζ) and was phospho-
rylated at Thr-410 to a similar extent as the wild type enzyme (Fig. 4B), consistent with the ability of this mutant to interact
with PDK1 (Fig. 2).

The Hydrophobic Motif of PKCζ Is Required for Phosphorylation of PKCζ and PRK2—Full-length PKR2 or ΔNT-PRK2 were expressed in 293 cells. The specific activity of full-length PRK2 was ~10-fold lower than ΔNT-PRK2 (Fig. 5A). As reported previously (32), incubation of full-length PRK2 with cardiolipin (25 μg/ml) increased its specific activity 5-fold, but this lipid did not further enhance the activity of ΔNT-PRK2 (data not shown). We prepared phospho-specific antibodies that only recognize PRK2 phosphorylated at Thr-816, the site of PDK1 phosphorylation (termed the T816-P antibody). This antibody recognized full-length PRK2 or ΔNT-PRK2 similarly, indicating that these proteins were phosphorylated equivalently at Thr-816 (Fig. 5B, middle panel). Incubation of the T816-P antibody with the phosphorylated phosphopeptide immunogen
used to raise the antibody (Fig. 5A, bottom panel), but not with the dephosphorylated peptide (Fig. 5A, middle panel), vastly reduced its ability to recognize full-length PRK2 or ΔNT-PRK2. Furthermore, a mutant form of ΔNT-PRK2 in which Thr-816 was changed to Ala was inactive (Fig. 5A) and was not recog-
nized by the T816-P antibody (Fig. 5B).

To study the role of the hydrophobic motif of PRK2 in mediating the phosphorylation of Thr-816 in PRK2, Phe-977, Glu-
978, and Tyr-979 in the hydrophobic motif of ΔNT-PRK2 were
individually mutated to Ala. The F977A-ΔNT-PRK2 mutant
was completely inactive, and no phosphorylation at Thr-816
was detected. The D978A-ΔNT-PRK2 and Y979A-ΔNT-PRK2
mutants were also significantly less active possessing only
~20% of the activity of the wild type enzyme and were phos-
phorylated at Thr-816 to a markedly lower extent than ΔNT-
PRK2 (Fig. 5C).

Overexpression of GST-PIF in Cells Prevents Phosphorylation and Activation of PKCζ and PRK2—PDK1 binds with
submicromolar affinity to a region of PRK2 encompassing its
hydrophobic motif, termed the PDK1-interacting fragment
(PIF) (29). The data presented thus far suggested that PDK1
when complexed to PIF would be unable to interact with and
phosphorylate PKCζ and PRK2. In order to test this hypothe-
sis, PKCζ full-length PRK2, and ΔNT-PRK2 were transfected
into 293 cells together with constructs encoding either GST-
PIF, a mutant form of GST-PIF that interacts with PDK1 very

FIG. 3. PRK2 C-terminal hydrophobic motif binds to the PIF-
binding pocket of PDK1. A and B, 293 cells were transiently trans-
fected with DNA constructs expressing wild type (wt) FLAG-PRK2 (A)
or FLAG-ΔNT-PRK2 (B) together with constructs expressing either
wild type or the indicated mutants of GST-PDK1. C, 293 cells were
transfected with DNA constructs expressing either GST, wild type, or
indicated mutants of GST-ΔNT-PRK2 together with wild type Myc-
PDK1. Binding is analyzed as described in the legend to Fig. 2. Dupli-
cates of each condition are shown. Similar results were obtained in at
least two separate experiments. The sequence of PRK2 C-terminal residues 972–980 comprising the hydrophobic motif is shown; Asp-978 lies at the equivalent position to Ser-473 in PKBα. The residues that were mutated are underlined.

FIG. 4. Disruption of the hydrophobic motif of PKCζ inhibits phosphorylation of Thr-410. 293 cells were transfected with con-
structs expressing FLAG epitope-tagged wild type (WT) or the indicated mutants of PKCζ. 36 h post-transfection the cells were lysed. PKCζ
immunoprecipitated, and either assayed for activity or subjected to
immunoblotting with the T410-P antibody as described under “Exper-
imental Procedures.” Cell lysates were also subjected to immunoblot-
ting with the FLAG antibody. Each experiment was carried out using
three separate dishes of cells for each condition. PKCζ was immuno-
precipitated in replicate from each dish and assayed. The activities
shown are the average ± S.D. for the three dishes of cells. Similar
results were obtained in three separate experiments performed on
different days.
weakly (GST-F977A-PIF (29)), or GST by itself. The wild type GST-PIF, the mutant GST-PIF, and GST were expressed at similar levels and were present at a much higher concentration than the endogenous PDK1, PKC\(\zeta\), or PRK2 (data not shown).

Expression of GST-PIF with PKC\(\zeta\) (Fig. 6A), full-length PRK2 (Fig. 6B), or ΔNT-PRK2 (Fig. 6C) greatly reduced the specific activity of these kinases, and their phosphorylation at the T-loop site compared with that observed when PKC\(\zeta\) and PRK2 were coexpressed with either GST or GST-F977A-PIF.

**DISCUSSION**

The hydrophobic motif of PRK2 (termed PIF) has previously been shown to interact directly with a pocket on the small lobe of the kinase domain of PDK1, termed the PIF-binding pocket (31). This suggested that the hydrophobic motif of PRK2 acts as a site where PDK1 binds prior to phosphorylating the T-loop site. In this paper, we demonstrate that C-terminal fragments of atypical PKC isoforms (PKC\(\zeta\) and PKCi) and the PKC-related kinases (PKR1 and PRK2) are capable of interacting with wild type PDK1 but not with mutant forms of PDK1 in which the conserved Leu-155, located in the PIF-binding pocket of PDK1 (31), has been altered. We demonstrate that mutation of the conserved aromatic residues of the hydrophobic motif of PKC\(\zeta\) and PRK2 not only reduces the affinity of these kinases for PDK1 but also inhibits the phosphorylation of PKC\(\zeta\) and PRK2 at their T-loop residue in cells. Furthermore, we observe that the activity of wild type and mutant PKC\(\zeta\) and PRK2 in cells correlates well with the degree of phosphorylation of these proteins at their T-loop motif (Figs. 4 and 5). This is consistent with previous studies suggesting that phosphorylation of these residues in PKC\(\zeta\) (13, 14, 28), PRK1, and PRK2 (15, 16) activates these kinases. Our findings indicate that the hydrophobic motifs of atypical and related PKC isoforms are likely to be

**Fig. 5.** Disruption of the hydrophobic motif of PRK2 inhibits phosphorylation of Thr-816. 293 cells were transfected with constructs expressing FLAG epitope tagged full-length (FL) PRK2, a mutant of PRK2 lacking the N-terminal regulatory domain (ΔNT) (A and B), or the indicated hydrophobic motif mutants of GST-ΔNT-PRK2 (C). 36 h post-transfection the cells were lysed, and PRK2 was immunoprecipitated and assayed for activity. Cell lysates were also subjected to immunoblotting with the T816-P antibody or the FLAG antibody. Each experiment was carried out using three separate dishes of cells for each condition. PRK2 was immunoprecipitated in triplicate from each dish and assayed. The activities shown are the average ± S.D. for the three dishes of cells. Similar results were obtained in three separate experiments performed on different days.

**Fig. 6.** PIF inhibits the activation and phosphorylation of PKC\(\zeta\) and PRK2. 293 cells were cotransfected with constructs expressing the FLAG-tagged wild type PKC\(\zeta\) (A), full-length PRK2 (B), or ΔNT-PRK2 (C) with either GST-PIF, GST-F977A-PIF, or GST. 36 h post-transfection the cells were lysed, and PKC\(\zeta\) or PRK2 was immunoprecipitated and assayed for activity. FLAG, T410-P, and T816-P immunoblots were carried out as described under "Experimental Procedures." Each experiment was carried out using three separate dishes of cells for each condition. PKC\(\zeta\)/PRK2 was immunoprecipitated in triplicate from each dish and assayed. The activities shown are the average ± S.D. for the three dishes of cells. Similar results were obtained in three separate experiments performed on different days.
acting as PDK1 docking sites analogous to those present in other kinases that are components of distinct kinase cascades such as mitogen-activated protein kinases, CDK2, and c-Jun N-terminal kinase (reviewed in Ref. 39).

The interaction of full-length PRK2, ΔNT-PRK2 (Fig. 3), or PIF itself (29) with PDK1 also requires an Asp residue (Asp-978) in the hydrophobic motif, at the position equivalent to Ser-473 of PKBα. Thus mutation of this residue to Ala greatly reduces affinity for PDK1. Consistent with this finding, the mutant D978A-PRK2 does not become phosphorylated at its T-loop when expressed in cells (Fig. 6). This confirms that Asp-978 is required for the recognition and phosphorylation of PRK2 by PDK1 in vivo. In contrast, mutation of the equivalent residue in the hydrophobic motif of PKCζ (Glu-579) to Ala does not affect the interaction of PKCζ with PDK1 significantly (Fig. 2). Consistent with this observation a mutant E579A-PKCζ expressed in 293 cells is still phosphorylated at its T-loop to a similar extent as the wild type enzyme (Fig. 5). Thus, for PKCζ, it appears that the aromatic residues of the hydrophobic motif are the key determinants that mediate binding to PDK1 (Fig. 2). In contrast, the aromatic residues and the acidic residue of the hydrophobic motif are both needed for the interaction of PRK2 with PDK1.

The E579A-PKCζ mutant possesses only marginally lower specific activity than the wild type protein (Fig. 5), indicating that a negative charge at this position is not critical for maximal activity. This is consistent with the finding that this mutation does not impair the ability to interact with PDK1. Phosphorylation of the hydrophobic motif of conventional PKC isoforms rather than activating these kinases serves to stabilize these enzymes in an active conformation (9, 40). It is possible that the conserved Glu-579 residue in the hydrophobic motif of PKCζ would play a similar role. Consistent with this idea, we find that the mutant E579A-PKCζ is significantly less stable than wild type PKCζ when heated. For example incubation of wild type PKCζ at 42 °C for 2 min reduces its activity by 50%, whereas the activity of the E579A-PKCζ mutant is reduced by over 80% under these conditions (data not shown).

PRK1 and PRK2 interact with activated members of the Rho GTPase family, which may activate and/or control the cellular location of these enzymes (15, 41–45). Full-length PRK1 and PRK2 possess a low specific activity that can be increased over 5-fold by the proteolysis of the N-terminal regulatory domain or by the interaction of PRK1 and PRK2 with acidic phospholipids such as cardiolipin (32, 46–48) and PtdIns(3,4,5)P3 (49). Rho complexed to GTP interacts with the N-terminal regulatory region of PRK1 and PRK2 (41), and recently the structure of Rho bound to this region of PRK1 has been solved (50). Thus far, no physiological substrates for the PRKs have been identified. However, PRK1 has been implicated in growth factor-induced cytoskeletal rearrangements (42), and PRK1 complexed to RhoB has recently been proposed to have a key role in regulating endocytic trafficking of the epidermal growth factor receptor (51).

Recent work has indicated that the interaction of PRK1 and PRK2 with Rho-GTP may enable these kinases to bind to PDK1 and so become phosphorylated at their T-loop residue (15). In contrast to this study, we were unable to demonstrate enhanced association of PDK1 with either full-length PRK2 or ΔNT-PRK2 or any increase in their phosphorylation at Thr-816 when these forms of PRK2 were cotransfected with a constitutively activated form of Rho (data not shown). It is possible that 293 cells already contain high levels of Rho-GTP, which could account for the high degree of T-loop phosphorylation of wild type PRK2 observed in our experiments. We were unable to reduce the level of Thr-816 phosphorylation of either full-length PRK2 or ΔNT-PRK2 in cells even after prolonged serum starvation of cells (data not shown). Furthermore, incubation of full-length PRK2 or ΔNT-PRK2 with very high levels of either protein phosphatase 1 or protein phosphatase 2A did not result in dephosphorylation of Thr-816 (or inactivation of the kinase). This suggests that once Thr-816 is phosphorylated by PDK1, it becomes buried in the protein and is thus inaccessible to protein phosphatases. A possible model is that when full-length PRK2 interacts with Rho-GTP through its N-terminal domain, it becomes capable of binding to PDK1 and is therefore phosphorylated at its T-loop. The phosphorylation of this residue results in a conformational change in which Thr-816 becomes inaccessible to protein phosphatases. It should be noted that the full-length PRK2 phosphorylated at Thr-816 is still capable of interacting with PDK1 in the absence of Rho-GTP (see Fig. 3).

Full-length PRK2 even when phosphorylated at Thr-816, in the absence of lipids such as cardiolipin, exists in a low activity conformation. It is likely that either lipids or regulatory proteins or PRK2 substrates will need to interact with the autoinhibitory non-catalytic N-terminal domain of PRK2 to enable activation of PRK2 in cells.

The overexpression of GST-PIF in cells prevented PDK1 from phosphorylating PKCζ and PRK2 (Fig. 6) and p70 S6K (30) at their T-loop site, but mutant forms of PIF that interacted weakly with PDK1 were much less effective at inhibiting the phosphorylation of PKCζ or PRK2 in cells (Fig. 6) as well as p70 S6K (30). These observations suggest that substrates for PDK1 such as p70 S6K, PKCζ, and PRK2 need to interact with PDK1 at a site that overlaps with the PIF-binding site, before they can become phosphorylated by PDK1. In contrast, we have not been able to demonstrate that PKB can interact with PDK1 in vitro (29) nor is the phosphorylation of PKB by PDK1 inhibited by the presence of PIF in vitro or in transfected 293 cells that have been stimulated with IGF1 (30). Because PKB and PDK1 both interact with 3-phosphoinositides via their pleckstrin homology domains, it is possible that this lipid second messenger is the primary mechanism for colocalizing these molecules at the plasma membrane, hence allowing PDK1 to phosphorylate PKB. In contrast, substrates of PKD1 that do not exhibit a high affinity interaction with 3-phosphoinositides, such as p70 S6K and atypical and related PKC isoforms, may actually need to form tight complexes with PDK1, before they can become phosphorylated.

Although it has been reported that PKCζ can be activated directly in vitro through its interaction with acidic phospholipids including PtdIns(3,4,5)P3 (52), there is a growing consensus that the activation of PI 3-kinase induces a moderate (2–3-fold) activation of PKCζ (53–55) that is mediated by the phosphorylation of PKCζ at its T-loop site by PDK1. PtdIns(3,4,5)P3 may enhance the rate of phosphorylation of this site (13, 14, 28). PKCζ has been suggested to play a role in regulating many cellular processes. However, the evidence for this is weak and largely based on overexpression of wild type PKCζ or catalytically inactive forms of this kinase. For example it has been reported that a catalytically inactive mutant of PKCζ when overexpressed in cells antagonizes the ability of agonists to activate p70 S6K (56), as well as other PKC isoforms (57). The results presented in this paper suggest that the overexpression of inactive forms of PKCζ or other atypical or related PKC isoforms in cells is likely to prevent PDK1 from interacting with and phosphorylating many of its protein kinase substrates. Thus great caution should be drawn in interpreting the results of such experiments.

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