Regulation of both PDK1 and the Phosphorylation of PKC-ζ and -δ by a C-Terminal PRK2 Fragment†

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ABSTRACT: The mechanism by which PDK1 regulates AGC kinases remains unclear. To further understand this process, we performed a yeast two-hybrid screen using PDK1 as bait. PKC-ζ, PKC-δ, and PRK2 were identified as interactors of PDK1. A combination of yeast two-hybrid binding assays and coprecipitation from mammalian cells was used to characterize the nature of the PDK1−PKC interaction. The presence of the PH domain of PDK1 inhibited the interaction of PDK1 with the PKCs. A contact region of PDK1 was mapped between residues 314 and 408. The interaction of PDK1 with the PKCs required the full-length PKC-ζ and -δ proteins apart from their C-terminal tails. PDK1 was able to phosphorylate full-length PKC-ζ and -δ but not PKC-ζ and -δ constructs containing the PDK1 phosphorylation site but lacking the C-terminal tails. A C-terminal PRK2 fragment, normally produced by caspase-3 cleavage during apoptosis, inhibited PDK1 autophosphorylation by >90%. The ability of PDK1 to phosphorylate PKC-ζ and -δ in vitro was also markedly inhibited by the PRK2 fragment. Additionally, generation of the PRK2 fragment in vivo inhibited by >90% the phosphorylation of endogenous PKC-ζ by PDK1. In conclusion, these results show that the C-terminal tail of PKC is a critical determinant for PKC-ζ and -δ phosphorylation by PDK1. Moreover, the C-terminal PRK2 fragment acts as a potent negative regulator of PDK1 autophosphorylation and PDK1 kinase activity against PKC-ζ and -δ. As the C-terminal PRK2 fragment is naturally generated during apoptosis, this may provide a mechanism of restraining prosurvival signals during apoptosis.

AGC kinases, such as protein kinase C (PKC),[1] protein kinase A (PKA), and protein kinase B (PKB), lie at the heart of cellular signaling (*1*−*4*) and are believed to be regulated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (*5*−*9*). However, the mechanism by which PDK1 regulates AGC kinases is still unclear (*10*−*17*). Signaling through several cellular PDK1 targets like PKC-ζ, PKB, and serum- and glucocorticoid-regulated protein kinase has been implicated as being necessary for cell survival and the prevention of apoptosis (*12*, *13*).

Protein kinase C-related kinase 2 (PRK2) belongs to a subclass of lipid and proteolysis-activated serine/threonine kinases that are highly homologous to PKCs in their catalytic domains (*18*). PRK2 is an effector of the small GTPases Rho (*19*, *20*) and Rac (*21*). In addition to this, PRK2 binds to the middle SH3 domain of the SH2−SH3 adaptor protein Nck (*20*), and consequently, PRK2 is predicted to be recruited to tyrosine-phosphorylated proteins that bind the Nck SH2 domain. PRK2 is cleaved by caspase-3 during apoptosis to generate the apoptotic C-terminal fragment of PRK2 (*22*, *23*). Very recently, this PRK2 fragment, but not full-length PRK2, has been shown to inhibit PKB activity (*16*), suggesting that the cleavage of PRK2 during apoptosis might act to inhibit prosurvival pathways leading to the amplification of proapoptotic signaling in the cell. However, the precise target of the PRK2 fragment remains unclear.

We addressed the question of how PDK1 regulates phosphorylation of AGC kinases via a yeast two-hybrid screen using PDK1 as bait. This yielded several interactors, PKC-ζ, PKC-δ, and PRK2. We found that the C-terminal tail of both PKC-ζ and -δ was not required for interaction with PDK1 but was critical for the phosphorylation of both of these PKCs by PDK1. In addition, the apoptotic C-terminal PRK2 fragment reduced the extent of phosphorylation of PKC-ζ and -δ by PDK1 and inhibited PDK1 autophosphorylation. This indicates that the apoptotic C-terminal PRK2 fragment negatively regulates signaling through PDK1 and the PKCs (ζ and δ) and as such identifies a novel control mechanism.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Assay. The LexA yeast two-hybrid system was purchased from Clontech. The human matchmaker brain cDNA library was also from Clontech. Full-length PDK1 was cloned from a rat brain library using the 5′ oligonucleotide $^{5'}$ATG GCC AGG ACC ACC AGC CAG CTG TAT GA$^{3'}$ and the 3′ oligonucleotide $^{5'}$TCA CTG AGC

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[1] Abbreviations: DAG, diacylglycerol; MK2, MAPKAP kinase 2; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol 3-kinase; PIF, PDK1-interacting fragment; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PRK, protein kinase C-related kinase.

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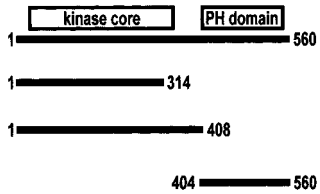

FIGURE 1: Schematic representation of the PDK1 constructs used in this study. Constructs were generated to cover the full-length portion (PDK1 1−560), the N-terminal domain (PDK1 1−314 and PDK1 1−408), and the C-terminal domain (PDK1 404−560) of the PDK1 protein.

RGC RTC YGG RTR GCT CT$^{3'}$ via Pfu polymerase. The constructs were A tailed and ligated into pGEM Teasy (Promega) and then sequenced. The inserts were then amplified via PCR via Pfu from this vector via the 5′ oligonucleotide $^{5'}$GCC GCC ATG GCG AAT TCG GCA ATT CGA$^{3'}$ and the 3′ oligonucleotide $^{5'}$GCA GGA GGC CGC CTC GAG ACT AGT GAT$^{3'}$. The PCR products, digested with *Eco*RI and *Xho*I, were then ligated into *Eco*RI−*Xho*I-digested pLexA to generate in-frame fusions with the LexA binding domain and confirmed by sequencing and expression of the full-length protein in yeast. The EGY48[p8opLacZ] yeast strain was first transformed with the pLexA−PDK1 fusion protein and tested for expression of the hybrid protein via Western blotting using the LexA antibody (Clontech). The pLexA−PDK1 fusion protein did not activate either the Leu or LacZ reporter. Subsequently, the EGY48[p8opLacZ, pLexA−PDK1] strain was transformed with the pB42AD-brain cDNA library (100 mg) and plated out onto SD/dex/kan/-his/-trp/-ura. After the cells had been grown for 3 days, the transformants were collected (5×10^6) and were plated out onto SD/gal/raf/kan/-his/-trp/-ura/-leu selection medium plates supplemented with X-gal (80 mg/mL). Leu+LacZ+ colonies were collected over a period of 5 days. Library plasmids were rescued via transformation of KC8 bacteria grown on M9TrpDOAmp plates. Putative interacting library plasmids were reintroduced into EGY48[p8opLacZ, pLexA−PDK1] as the positive control and with pLexA-laminin and pLexA-p53 as negative controls with the selection on SD/gal/raf/kan/-his/-trp/-ura/-leu/X-gal plates.

PDK Deletion Mutant Bait Plasmids. PDK fragment 1−314 was constructed by digesting the pLexA−PDK1 fusion protein with *Eco*RI and *Bgl*II. This was ligated into *Eco*RI−*Bam*HI-digested pLexA. PDK1 fragment 1−408 was produced by PCR with Pfu polymerase of the pLexA−PDK1 fusion with the 5′ oligonucelotide $^{5'}$GCC GCC ATG GCG AAT TCG GCA ATT GCA$^{3'}$ and the 3′ oligonucleotide $^{5'}$GGG GCA GGC TGG CTC GAG CCG CAC A$^{3'}$. PDK1 fragment 404−560 was made via the 5′ oligonucleotide $^{5'}$CCG ACA CGG GCC TGC CCG AAT TCT CAG GCA GCA ACA TA$^{3'}$ and the 3′ oligonucleotide $^{5'}$GCA GGA GGC CGC CTC GAG ACT AGT GAT$^{3'}$. Constructs were digested with *Eco*RI and *Xho*I and ligated into *Eco*RI−*Xho*I-digested pLexA. These constructs are shown in Figure 1.

PKC Deletion Mutant Prey Plasmids. Full-length PKC-ζ was isolated as one of the positives of the yeast two-hybrid screen in this paper. This served as the basis for PCR, using Pfu polymerase, in generating various PKC-ζ deletion mutants.

Full-length PKC-δ and deletion mutants were generated by PCR, using Pfu polymerase, from the brain cDNA library and from the positives isolated from the yeast two-hybrid screen.

Table 1: PDK1 Interacts with PKC-ζ and -δ in Yeast[a]

PKC construct	PDK1	PKC construct	PDK1
PKC-ζ	+	PKC-δ	+
PKC-ζ −37−574	+	PKC-δ 1−448	−
PKC-ζ −37−408	−	PKC-δ 454−602	−
PKC-ζ 394−574	−	PKC-δ 500−626	+
PKC-ζ 516−592	+		

[a] The yeast reporter strain was cotransfected with PDK1 and various PKCs. Transformant yeast were assayed for growth on leucine deficient medium and for the appearance of blue color for up to 5 days. + refers to the Leu+LacZ+ phenotype and represents a two-hybrid interaction. − refers to no interaction.

For a complete listing of constructs, see Table 1. All oligonucleotides are shown with the forward primer first and then the reverse primer.

PKC-ζ constructs: −37−574 from full-length PKC-ζ, $^{5'}$GGT TTT CAT GAA TTC GAA GCG GAT$^{3'}$ $^{5'}$CTC TGA CTG GTC GAT CTC GAG TAT GGC ATC CTC AT$^{3'}$; −37−408 from full-length PKC-ζ, $^{5'}$GGT TTT CAT GAA TTC GAA GCG GAT$^{3'}$ $^{5'}$CGT TGT GTC ACC AGG CTC GAG GCC TTC CTT GAA$^{3'}$; and 394−574 from the yeast two-hybrid clone from this screen, $^{5'}$GGT TTT CAT GAA TTC GAA GCG GAT$^{3'}$ $^{5'}$CTC TGA CTG GTC GAT CTC GAG TAT GGC ATC CTC AT$^{3'}$. All other PKC-ζ constructs were from the screen.

PKC-δ constructs: 1−626, $^{5'}$GCG ACC ATG TAT CCT GAA TTC AAG TCG ACG TT$^{3'}$ $^{5'}$GCT AGC CTG ATC TCG AGA GGA ACC TCA ATC AA$^{3'}$; 1−602, $^{5'}$GCG ACC ATG TAT CCT GAA TTC AAG TCG ACG TT$^{3'}$ $^{5'}$GC AGA CTG GTC CAT GCT CGA GAT GAG GTT CTT$^{3'}$; 1−448, $^{5'}$GCG ACC ATG TAT CCT GAA TTC AAG TCG ACG TT$^{3'}$ $^{5'}$ CCG GCT CTC CCC CTC GAG GTT CTC TTT GCA CAT$^{3'}$; 454−626, $^{5'}$GAG AAC ATA TTC GGG GAA TTC CGG GCC AGC ACC T$^{3'}$ $^{5'}$GCT AGC CTG ATC TCG AGA GGA ACC TCA ATC AA$^{3'}$; and 454−602, $^{5'}$GAG AAC ATA TTC GGG GAA TTC AGG GCC AGC ACC T$^{3'}$ $^{5'}$GC AGA CTG GTC CAT GCT CGA GAT GAG GTT CTT$^{3'}$. PKC-δ 500−626 was from the two-hybrid screen.

All constructs were digested with *Eco*RI and *Xho*I and ligated into *Eco*RI−*Xho*I-digested pB42AD.

HA- and cMyc-Tagged pcDNA Constructs. pLexA and pB42AD constructs served as templates for Pfu polymerase-dependent PCR in generating myc- and HA-tagged constructs listed above. pcDNA3.1+ (Invitrogen) was the mammalian expression vector that was used. Prior to ligation, the vector was digested with *Kpn*I and *Xba*I. Myc-tagged fusion constructs were generated via the 5′ oligonucleotide $^{5'}$AAC AAT TGT CGT AGG TAC CGG TAT GGA GGA GCA GAA GCT GAT CTC AGA GGA CCT GGT TAT TCG CAA CGG CGA CTG GCT G$^{3'}$ and the 3′ oligonucleotide $^{5'}$ACC TAA GAG TCA CTT CTA GAT TTG TAT ACA CT$^{3'}$ as suggested by Clontech. This PCR was very inefficient; only one insert could be successfully amplified. This was digested with *Kpn*I and *Xba*I and ligated into the predigested pcDNA3.1. Once sequenced, this vector was cut with *Eco*RI and *Xho*I to generate a pcDNAmyc vector. pLexA vectors containing the remaining constructs were cut with *Eco*RI and *Xho*I to give rise to the insert, which was rescued from gels via a Qiagen gel extraction kit. The

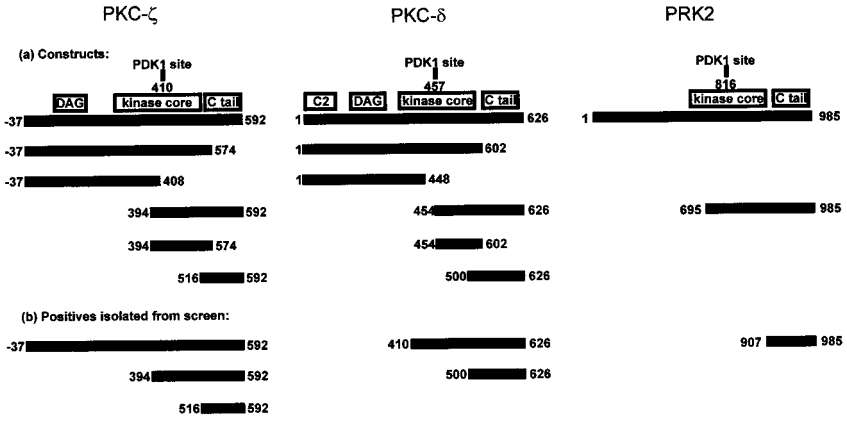

FIGURE 2: Schematic representation of the PKC-ζ, PKC-δ, and PRK2 constructs used in this study. (a) Constructs that were used. The position of the PDK1 site is indicated for PKC-ζ, PKC-δ, and PRK2. (b) Regions of the PKC-ζ, PKC-δ, and PRK2 proteins isolated in the yeast two-hybrid screen of the brain cDNA library.

subsequent DNA was then ligated into pcDNAmyc. HA-tagged fusion proteins were generated by PCR using Pfu with the 5′ oligonucelotide $^{5'}$GTT AAC GAT ACC AGC GGT ACC CTG AGT ATG GAT GC$^{3'}$ and the 3′ oligonucleotide $^{5'}$ACC TAA GAG TCA CTC TAG AAT TTG TAT ACA CT$^{3'}$. The catalytic domain of PRK2 was cloned into an *Eco*RI−*Xho*I-digested pcDNA-HA vector after PCR from a brain library using the primers $^{5'}$CGA GAT GAA TTC GAC AGC CT$^{3'}$ and $^{5'}$GCA GTG TCT CTC GAG TTA ACA CCA$^{3'}$. This generated a PRK2 fragment that encompassed amino acids 695−985 of the PRK2 protein.

Transient Cell Transfection and Immunoprecipitation. Exponentially growing cos-1 cells (2×10^6) were transiently transfected with plasmid combinations as listed in the figure legends using the effectene transfection reagent according to the manufacturer's protocol (Qiagen). After 48 h, cell lysates were prepared using lysis buffer [0.14 M NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 5 mM EDTA, 0.5% (v/v) Triton X-100, 0.1 mM PMSF, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, and 25 μg/mL aprotinin (pH 7.4)]. Lysis was allowed to proceed for 30 min on ice, after which the cell debris was removed by centrifugation at 12000*g* for 5 min. Extracts were used immediately for immunoprecipitation. Extracts were diluted in lysis buffer to give a final volume of 50 μL (500 μg) and then further diluted in coimmunoprecipitation buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 5 μg/mL aprotinin, 0.5 mM PMSF, and 0.1% (v/v) Tween 20 (pH 7.4, 400 μL)] with HA (4 μg, Sigma) or cMyc (clone 4E910, 4 μg, Clontech) antibodies and protein G beads (20 μL, Sigma). After continuous gentle agitation at 4 °C for 6 h, the beads were collected by pulse spin and then washed three times in coimmunoprecipitation buffer, after which they were resuspended in PBS. Immunoprecipitates were resolved (*24*, *25*) by SDS−PAGE and transferred to a nitrocellulose filter. The membranes were then probed with the monoclonal HA or cMyc antibody followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) or the phospho T-loop antibody (New England Biolabs) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) to determine the level of phosphorylation of the PKCs at the PDK1 site. Blots were developed with the ECL system according to the manufacturer's instructions (Amersham).

In Vitro Kinase Assays. In vitro kinase assays using cos-1 immunoprecipitated PDK1 and various PKC-ζ and -δ constructs were carried out at 30 °C for 30 min in a buffer containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 400 μM sphingosine (*26*), 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP per assay (*27*). The PKC activators phosphatidylserine (200 μg/mL) and diacylglycerol [DAG (1,2-dioctanoyl-*sn*-glycerol), 40μg/mL] were added to the kinase reaction mixture where indicated. The reactions were stopped by addition of 5× Laemmli sample buffer and subsequent boiling for 3 min. Samples were then subjected to 7.5% SDS−PAGE, transferred to nitrocellulose, and exposed to Amersham HybondN film at −80 °C. Lysates were prepared using the lysis buffer described above with the addition of 50 mM NaF, 40 mM β-glycerol phosphate, 10 mM Hepes (pH 7.3), 5 mM EDTA, and 1 μM microcystin. All transfections were serum starved for 24 h prior to extraction and immunoprecipitation. Immunoprecipitation was carried out as described above except that the beads, after being washed in coimmunoprecipitation buffer three times, were washed an additional two times in kinase buffer lacking ATP.

RESULTS

Detection of Proteins Binding to PDK1. To identify proteins that are able to interact with PDK1, we employed the LexA yeast two-hybrid system to screen a human brain cDNA library. Full-length rat PDK1 was fused to the LexA DNA binding domain of the Clontech pLexA vector and subsequently used in an interactor hunt with a brain cDNA library. Screening of 5×10^6 transformants yielded 59 Leu+LacZ+ colonies. Sequencing of the clones yielded full-length and partial sequences of PKC-ζ, and the C-terminal portions of PKC-δ and PRK2. These are shown in Figure 2b. The full-length PKC-ζ isolated from the screen had an additional 37 amino acids at the N-terminus compared to published sequences.

PDK1 Interaction with PKC-ζ and -δ in the Yeast Two-Hybrid System. To determine where PDK1 interacts with PKC-ζ and -δ in yeast, we prepared a series of PKC constructs (Figure 2a) which were cotransfected into yeast. All constructs were expressed at similar levels in yeast as determined by Western blotting with LexA and HA antibodies (data not shown). PDK1 interacted with PKC-ζ, PKC-ζ

Table 2: Mapping the Domains of PDK1 Responsible for the Interaction with PKC-ζ and -δ in Yeast[a]

PDK1 construct	PKC-ζ	PKC-δ	PDK1 construct	PKC-ζ	PKC-δ
PDK1	+	+	PDK1 1−314	−	−
PDK1 1−408	+	+	PDK1 404−560	−	−

[a] The yeast reporter strain was cotransfected with PKC-ζ or -δ and various PDKs. Transformant yeast were assayed for growth on leucine deficient medium and for the appearance of blue color for up to 5 days. + refers to the Leu+LacZ+ phenotype and represents a two-hybrid interaction. − refers to no interaction.

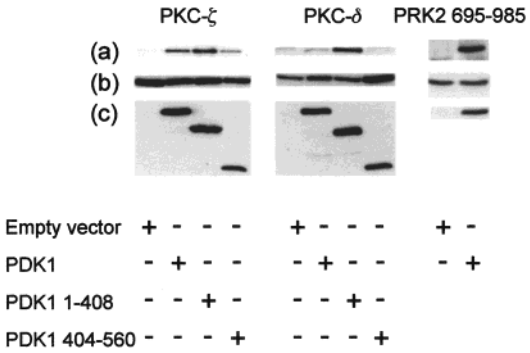

FIGURE 3: PDK1 interacts with PKC-ζ, PKC-δ, and the C-terminal PRK2 fragment in cos-1 cells. Cos-1 cells were transiently transfected as indicated with the PDK1 constructs (expressed as cmyc fusions) and PKCs or the C-terminal PRK2 fragment (expressed as HA fusions). Cell extracts were immunoprecipitated with the cmyc antibody, and after several washes, proteins were separated by 7.5% SDS−PAGE, transferred to a nitrocellulose membrane, and probed for (a) HA to detect coprecipitated proteins and (c) cmyc to check for immunoprecipitation of the PDK1 constructs. Aliquots of the lysate were also analyzed for levels of expression of HA-tagged PKC-ζ, PKC-δ, or the C-terminal PRK2 fragment using anti-HA antibodies (b). These immunoblots are representative of nine individual experiments.

−37−574, PKC-ζ 516−592, PKC-δ, and PKC-δ 500−626 (Table 1). No interaction was seen with those constructs that contained the PDK1 phosphorylation site only, namely, PKC-ζ 394−574 and PKC-δ 454−602 (Table 1).

The yeast system was then used to determine which region of PDK1 was responsible for the interaction with PKC-ζ and -δ (Table 2). When the full catalytic domain was expressed (PDK1 1−408), interaction with both PKC-ζ and -δ was seen. However, truncating the PDK1 1−408 construct by 88 amino acids (PDK1 1−314) removed the interaction with PKC-ζ and -δ. The PDK1 construct encompassing the PH domain (PDK1 404−560) failed to show any interaction with either PKC-ζ or -δ. These results map a region of PDK1 that is critical for interaction between residues 314 and 408.

PDK1 Interacts with PKC-ζ and -δ in Mammalian Cos-1 Cells. To test whether PDK1 and PKC-ζ and -δ interact in vivo in mammalian cells, cmyc-tagged PDK1 and HA-tagged PKC-ζ or -δ were cotransfected into cos-1 cells. Transfected cells were lysed, and the lysates were incubated with cmyc antibodies to precipitate cmyc-tagged PDK1. The immunoprecipitates were then separated by SDS−PAGE and immunoblotted with anti-HA antibodies to test for coprecipitation of HA-tagged PKC-ζ or -δ. As shown in Figure 3a, precipitation of PDK1 resulted in the coimmunoprecipitation of PKC-ζ and -δ (lanes 2 and 6), confirming our above-described yeast two-hybrid binding results. That this association was not a result of nonspecific binding is evident from the observation that PKC-ζ or -δ, when transfected alone, was not precipitated by the cmyc antibodies (Figure 3a, lanes 1 and 5). We also employed a C-terminal PRK2 fragment (residues 695−985) known to be produced by caspase-3 cleavage during apoptosis (*16*, *22*, *23*). This fragment interacted strongly with PDK1 (Figure 3a, lanes 9 and 10).

To determine which regions of PDK1 were responsible for the interaction with PKC-ζ and -δ, various cmyc-tagged deletion mutants of PDK1 (see Figure 1) were cotransfected into cos-1 cells with HA-tagged PKC-ζ or -δ. Transfected cells were lysed and the cmyc-tagged PDK1 constructs immunoprecipitated and probed for PKC-ζ or -δ with the HA antibody. The N-terminal domain of PDK1 (PDK1 1−408) was found to associate with PKC-ζ and -δ (Figure 3a, lanes 3 and 7), whereas PDK1 404−560 did not interact with PKC-δ (Figure 3a, lane 8) and exhibited only a slight interaction with PKC-ζ (Figure 3a, lane 4). PDK1 1−408, which lacks the region encompassing the PH domain, was found to coimmunoprecipitate significantly more PKC-ζ and -δ (Figure 3a, lanes 3 and 7) than the full-length PDK1 protein (Figure 3a, lanes 2 and 6). This was more marked with PKC-δ. PDK1 was expressed at a level similar to that of PDK1 1−408 and PDK1 404−560 (Figure 3c). Therefore, the interaction between PDK1 1−408 and PKC-ζ or -δ was stronger than that for wild-type PDK1. We could not express the PDK1 1−314 construct in cos-1 cells.

Experiments were then conducted to investigate the domain(s) within PKC-ζ and -δ required for binding to PDK1. Cos-1 cells were transiently transfected with the cmyc-tagged PDK1 along with various HA-tagged deletion mutants of PKC-ζ and -δ (see Figure 2a for details of the constructs). Transfected cells were lysed, immunoprecipitated with the HA antibody, and probed with the cmyc antibody (Figure 4a). A strong interaction was observed with full-length PKC-ζ and -δ (Figure 4a, lanes 2 and 7, respectively) and PKC constructs lacking the C-terminal tail, namely, PKC-ζ −37−574 and PKC-δ 1−602 (Figure 4a, lanes 3 and 8, respectively). We were unable to express those PKC constructs that contained only the C-terminal tail, namely, PKC-ζ 516−592 and PKC-δ 500−626. Again, as we showed in yeast, PDK1 did not interact with PKC constructs containing only the PDK1 binding site, namely, PKC-ζ 394−592, PKC-ζ 394−574, and PKC-δ 454−602 (Figure 4a, lanes 5, 6, and 10, respectively).

The C-Terminal PRK2 Fragment Inhibits PDK1 Autophosphorylation. PDK1 has been reported to undergo significant autophosphorylation in in vitro kinase assays (*5*, *17*, *26*). Indeed, in our system, we also found this to be the case (Figure 5a, lanes 1 and 3, and Figure 5d, bar 1). We found that the C-terminal PRK2 fragment inhibited the autophosphorylation of PDK1 by >90% (Figure 5a, lane 2, and Figure 5d, bar 2). PKC-ζ and -δ also inhibited PDK1 autophosphorylation, though to a significantly lesser degree than the C-terminal PRK2 fragment (Figure 5a, lanes 4 and 6, and Figure 5d). PKC constructs lacking the C-terminal tail, namely, PKC-ζ −37−574 and PKC-δ 1−602, also similarly reduced the extent of PDK1 autophosphorylation (Figure 5a, lanes 5 and 7, and Figure 5d).

PDK1 Phosphorylation of PKC-ζ and PKC-δ Requires the C-Terminal Tail of the PKCs. In vitro kinase assays using immunoprecipitated PKC-ζ, PKC-δ, PDK1, and [γ-^{32}P]ATP

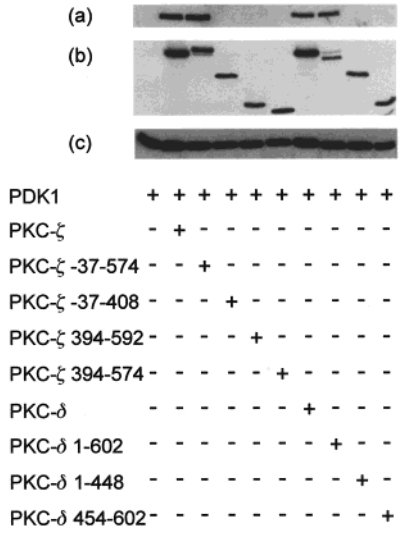

FIGURE 4: PDK1 interaction with PKC-ζ and -δ does not require the C-terminal tail of either PKC. Cos-1 cells were transiently transfected with cmyc-tagged PDK1 either alone or in combination with various HA-tagged PKC constructs. Cell extracts were immunoprecipitated with the HA antibody, and after several washes, proteins were separated by 7.5% SDS−PAGE, transferred to a nitrocellulose membrane, and probed for (a) cmyc to detect coprecipitated proteins and (b) HA to determine the levels of immunoprecipitation of the HA constructs. The PKC-ζ −37−574 construct consistently ran at a higher molecular weight than PKC-ζ. The cell extracts were probed for cmyc to check the expression of PDK1 (c). These immunoblots are representative of nine individual experiments.

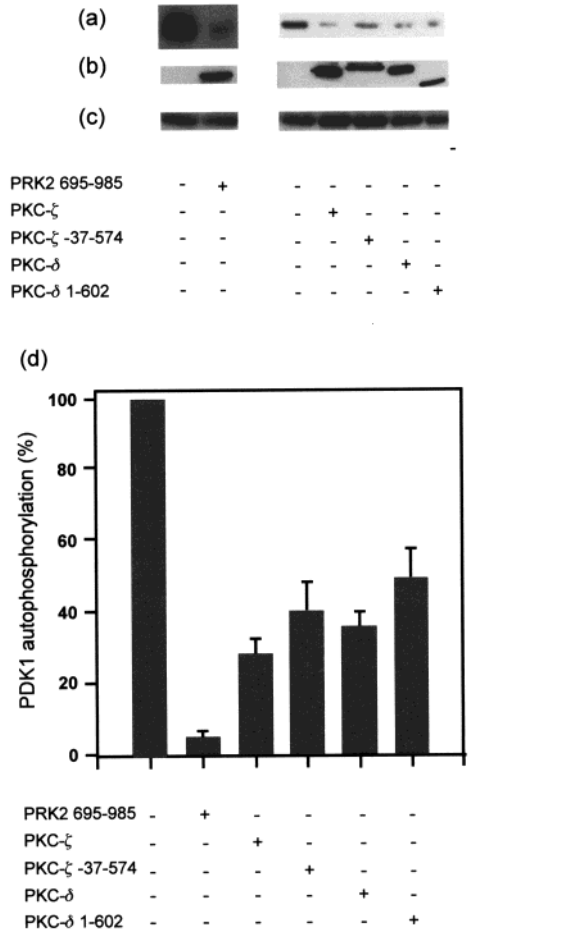

FIGURE 5: PDK1 autophosphorylation is reduced by the C-terminal PRK2 fragment and PKCs-ζ and -δ. PDK1 (cmyc-tagged) and PKCs or the C-terminal PRK2 fragment (HA-tagged) were isolated by immunoprecipitation from serum-starved (24 h) cos-1 cells transiently transfected with the respective constructs. PDK1 was incubated with [γ-^{32}P]ATP either alone or in combination with the various PKCs or the C-terminal PRK2 fragment as indicated. Samples were resolved by 7.5% SDS−PAGE and proteins transferred to nitrocellulose. The filters were exposed to X-ray film at −80 °C for 24 h and also immunoblotted with the various antibodies. (a) Representative autoradiographs showing PDK1 autophosphorylation. The nitrocellulose filters were further probed with the HA antibody (b) to determine the levels of HA-tagged PKC or PRK2 constructs and subsequently with the cmyc antibody (c) to determine the levels of cmyc-tagged PDK1. (d) Quantification of PDK1 phosphorylation. The level of PDK1 autophosphorylation was determined by densitometric scanning of the autoradiographs. Results are expressed as a percentage of PDK1 alone and are the means ± standard error of the mean of five independent experiments.

were carried out to determine whether PKC-ζ and -δ are substrates for PDK1. Both PKC-ζ and -δ were significantly phosphorylated by PDK1 (Figure 6a, bars 3 and 4, and Figure 6b, lanes 3 and 4). The phosphorylation of PKC-ζ and -δ was abolished if the truncated PKC constructs, PKC-ζ −37−574 and PKC-δ 1−602, were used instead (Figure 6a, bars 7 and 8, and Figure 6b, lanes 7 and 8, respectively). To test whether the increase in the level of ^{32}P incorporation into PKC-ζ and -δ reflected activation loop phosphorylation, samples were blotted with a phospho T-loop antibody which detects phosphorylated threonines in the activation loop. Some phosphorylation of the activation loop site of PKC-ζ and -δ was detected following incubation in the absence of exogenously added PDK1 (Figure 6c, lanes 1 and 2). This appeared to be due to coisolation of some endogenous PDK1 (see below). Nonetheless, addition of exogenous PDK1 increased the level of phosphorylation of the activation loop sites 2−4-fold, based on quantification of the blots by densitometric scanning (Figure 6c, lane 1 vs lane 3 for PKC-ζ and lane 2 vs lane 4 for PKC-δ). This was less than the >10-fold increase in the level of ^{32}P incorporation observed upon addition of PDK1 (Figure 6a, bars 3 and 4, and Figure 6b, lanes 3 and 4). This is presumably due to the robust autophosphorylation activity that PKC is known to exhibit. PDK1 did not phosphorylate the activation loop site of the truncated PKC constructs, PKC-ζ −37−574 and PKC-δ 1−602 (Figure 6c, lanes 7 and 8, respectively). Inclusion of the PKC activators, phosphatidylserine and DAG, which are required for optimal activation of the PKCs (*28*) had no effect on the ability of PDK1 to phosphorylate the activation loop site of PKC-ζ and -δ or on the inability of PDK1 to phosphorylate the activation loop site of the truncated PKC constructs, PKC-ζ −37−574 and PKC-δ 1−602 (Figure 7a,d). Control experiments confirmed that the cofactors activated the PKCs (not shown).

The C-Terminal PRK2 Fragment Abrogates PDK1 Phosphorylation of PKC-ζ and -δ. The effect of the C-terminal PRK2 fragment on the ability of PDK1 to phosphorylate PKC-ζ and -δ was next tested. This PRK2 construct was found to significantly reduce the level of phosphorylation of PKC-ζ by PDK1 in vitro (Figure 8a, bars 7 and 8). Additionally, phosphorylation of the activation loop of PKC-ζ by PDK1 was markedly inhibited by the C-terminal PRK2 fragment (Figure 8b, lanes 7 and 8). Similar inhibitory effects of the PRK2 construct were obtained when PKC-δ

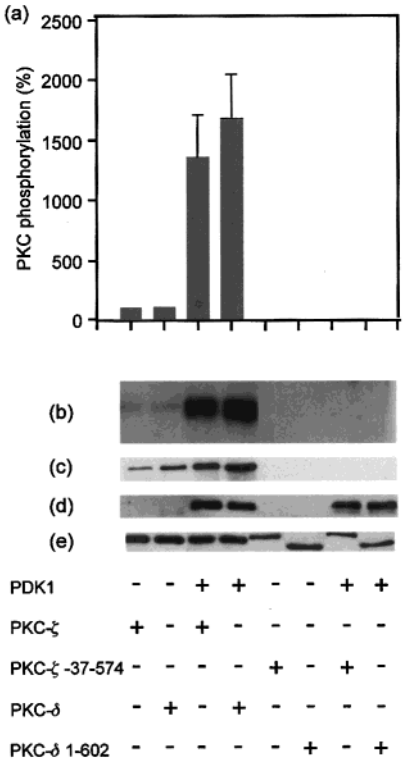

FIGURE 6: PDK1 is unable to phosphorylate PKC-ζ or -δ constructs lacking the C-terminal tail. PDK1 (cmyc-tagged) and PKCs (HA-tagged) were isolated by immunoprecipitation from serum-starved (24 h) cos-1 cells transiently transfected with the respective constructs. PDK1 was incubated with [γ-^{32}P]ATP and the various PKCs as indicated. Samples were resolved by 7.5% SDS−PAGE and the proteins transferred to nitrocellulose. The filters were exposed to X-ray film at −80 °C for 6 h and also immunoblotted with the indicated antibodies. (a) Quantification of PKC phosphorylation. ^{32}P incorporation into PKC was assessed by densitometric scanning of the autoradiographs. Results are expressed as a percentage of wild-type PKC alone and are the mean ± the standard error of the mean of five independent experiments. (b) A representative autoradiograph showing PKC phosphorylation. The filter was further probed with the phospho T-loop antibody (c) to determine the level of phosphorylation of the activation loop of the PKCs, the cmyc antibody (d) to determine the levels of cmyc-tagged PDK1, and the HA antibody (e) to determine the levels of the HA-tagged PKC constructs. Densitometric scanning showed that PDK1 increased the level of phosphorylation of the activation loop of PKC-ζ and -δ 4- and 2.2-fold, respectively.

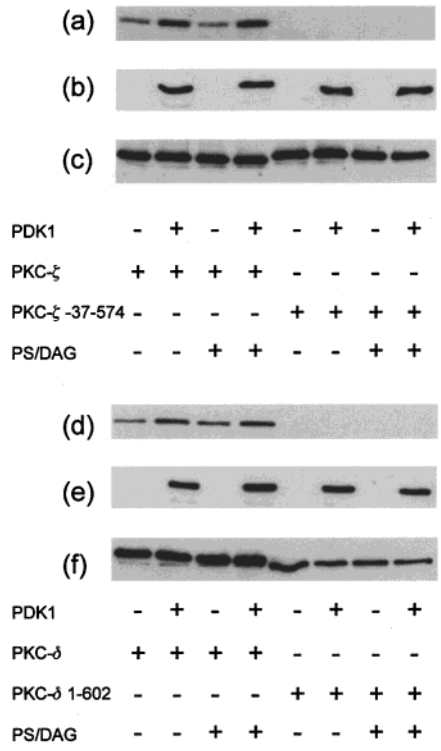

FIGURE 7: Effect of PKC activators on the phosphorylation of PKC-ζ and -δ by PDK1. PDK1 (cmyc-tagged) and PKCs (HA-tagged) were isolated by immunoprecipitation from serum-starved (24 h) cos-1 cells transiently transfected with the respective constructs. PDK1 was incubated with ATP and the various PKCs as indicated in the presence or absence of the PKC activators phosphatidylserine (PS) and DAG. Samples were resolved by 7.5% SDS−PAGE and the proteins transferred to nitrocellulose. The filter was probed with the phospho T-loop antibody **(**a and d) to determine the level of phosphorylation of the activation loop of the PKCs, the cmyc antibody (b and e) to determine the levels of cmyc-tagged PDK1, and the HA antibody (c and f**)** to determine the levels of the HA-tagged PKC constructs. The immunoblots are representative of two independent experiments.

was used as the substrate for PDK1 (Figure 8a, bars 9 and 10, and Figure 8b, lanes 9 and 10). As previously noted, some phosphorylation of the activation loop site of PKC-ζ and -δ was evident following incubation in the absence of exogenously added PDK1 (Figure 8b, lanes 2 and 5). Because little activation loop phosphorylation was apparent when the starting PKCs were directly analyzed without incubation (Figure 8b, lanes 1 and 4), this activation loop phosphorylation appears to be mainly due to coisolation of some endogenous PDK1.

It was important to determine whether the C-terminal PRK2 fragment inhibited PDK1 action in vivo. To test this, the PRK2 fragment was generated in intact cells. Transfection of PDK1 alone into cos-1 cells significantly increased the level of phosphorylation of the activation loop of endogenous PKC-ζ when compared with that of untransfected cells. Transfection of the C-terminal PRK2 fragment along with PDK1 resulted in a >90% inhibition of the phosphorylation of the activation loop of endogenous PKC-ζ (Figure 9a, compare lanes 2 and 3). Expression of the C-terminal PRK2 fragment also inhibited by >90% the phosphorylation of the activation loop of endogenous PKC-ζ in the absence of expressed PDK1 (Figure 9a, compare lanes 1 and 4).

DISCUSSION

In this study, we have investigated how two AGC kinases, PKC-ζ and -δ, interact with and are phosphorylated by PDK1. The role of an apoptotic C-terminal fragment of PRK2 in regulating both autophosphorylation of PDK1 and phosphorylation of PKC-ζ and -δ by PDK1 was also examined.

We found that PDK1 interacted with PKC-ζ and -δ via the N-terminal region of PDK1, which encompassed the catalytic domain, in both yeast and mammalian cells. The interaction between the N-terminal PDK1 construct, PDK1 1−408, and PKC-ζ and -δ was much stronger than that between wild-type PDK1 and PKC-ζ and -δ. PDK1 1−408 lacks the C-terminal PH domain of PDK1 and as such implies that the PH domain was inhibitory for the PDK1 interaction with PKC-ζ and -δ. Though PH domains bind to phospholipids such as PIP_3 and PIP_2 (*29*), there is ample evidence to

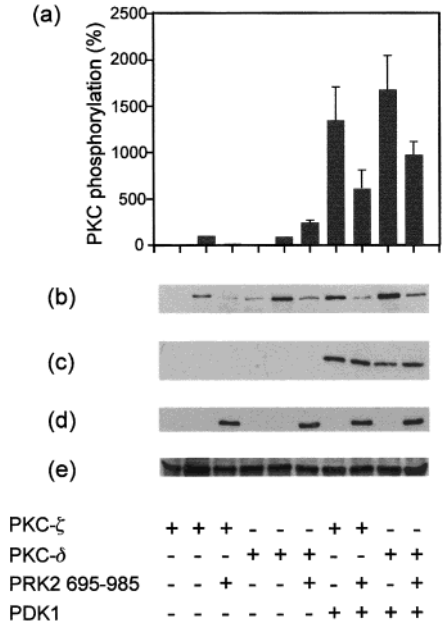

FIGURE 8: C-Terminal PRK2 fragment reduces the level of PDK1 phosphorylation of both PKC-ζ and -δ. PDK1 (cmyc-tagged), PKCs (HA-tagged), and the C-terminal PRK2 fragment (HA-tagged) were isolated by immunoprecipitation from serum-starved (24 h) cos-1 cells transiently transfected with the respective constructs. PDK1 was incubated with [γ-^{32}P]ATP, the various PKCs as indicated, and the C-terminal PRK2 fragment where appropriate. In addition, the PKCs were directly analyzed without any treatment or incubation (lanes 1 and 4). Samples were resolved by 7.5% SDS−PAGE and proteins transferred to nitrocellulose. The filters were exposed to X-ray film at −80 °C for 6 h. (a) Quantification of PKC phosphorylation. ^{32}P incorporation into PKC was assessed by densitometric scanning of the autoradiographs. Results are expressed as a percentage of the wild-type PKC without PRK2 and are the means ± the standard error of the mean of three independent experiments. A representative filter was probed with the phospho T-loop antibody to determine the level of phosphorylation of the activation loop of the PKCs (b), the cmyc antibody to determine the levels of cmyc-tagged PDK1 (c), and the HA antibody to determine the levels of the HA-tagged C-terminal PRK2 fragment (d) and the HA-tagged PKCs (e).

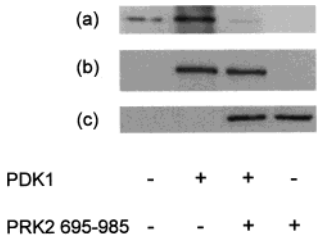

FIGURE 9: C-Terminal PRK2 fragment inhibits the phosphorylation of the activation loop of PKC-ζ by endogenous or expressed PDK1 in vivo. Cos-1 cells were transiently transfected with or without cmyc-tagged PDK1 and the HA-tagged C-terminal PRK2 fragment as indicated and serum-starved for 24 h before extraction. Cell extracts were immunoprecipitated with a PKC-ζ antibody to isolate endogenous PKC-ζ. After the immunoprecipitates had been extensively washed, proteins were resolved by 7.5% SDS−PAGE, transferred to nitrocellulose membrane, and probed with the phospho T-loop antibody to determine the level of phosphorylation of the activation loop of endogenous PKC-ζ (a), the cmyc antibody to determine the levels of cmyc-tagged PDK1 (b), and the HA antibody to determine the levels of the HA-tagged PRK2 construct (c). The immunoblots are representative of two individual experiments.

suggest that they are also involved in regulating protein interactions in both a positive (*30*−*36*) and negative fashion (*37*, *38*). These inhibitory effects of PH domains appear to rely on an intramolecular mechanism. Vav and Sos1, Dbl family guanine nucleotide exchange factors, which activate Rho family GTPases such as Rac in response to PI3K products, require the disruption of inhibitory intramolecular interactions involving the PH domain and the Dbl homology domains to activate Rac. This disruption of the PH−Dbl interaction is accomplished by PIP_3 (*37*). In a similar fashion, the PH domain of the Ras-p120 GTPase-activating protein (Ras-GAP) binds to the catalytic domain of Ras-GAP and interferes with the interaction with Ras via competitive intramolecular binding (*38*). Recently, it has been shown that deletion of the PH domain from PDK1 stimulated PDK1 autophosphorylation in vitro, suggesting that the PH domain of PDK1 blocks the catalytic activity of PDK1 under basal conditions (*17*). However, no work on whether the PH domain of PDK1 plays a role in mediating PDK1 interactions with other proteins has been carried out. Our study has shown that the PH domain of PDK1 is inhibitory with respect to interaction with PKC-ζ and -δ. This raises the possibility that when the PH domain of PDK1 binds to phospholipids, such as PIP_3, in response to extracellular stimuli, this could significantly enhance the interaction between PDK1 and PDK1 substrates. The PH domain may also be involved in determining the substrate specificity of PDK1 as the removal of the PH domain from PDK1 enhanced the interaction with PKC-δ to a much larger extent than that for PKC-ζ.

However, despite the inhibitory effect we have seen with the PH domain of PDK1, our results do not exclude the possibility that there is some interaction between the PH domain of PDK1 and the PKCs. Thus, the PDK1 404−560 construct which corresponds to the PH domain of PDK1 coprecipitated a small amount of PKC-ζ from cos-1 cells. As this interaction was not detected in yeast, this would suggest that it is rather weak. Although the level of the interaction between the PDK1 PH domain and PKC-ζ is low, it is difficult to rule out the possibility that it is significant solely on the basis of these types of experiments. For example, the PH domain of Bruton's tyrosine kinase has been shown to interact with the C1 domain of PKC (*34*). It is possible, therefore, that the PH domain mediates a complex array of interactions, the outcome of which may depend, for example, on the occupancy of phosphorylation sites and presence or absence of lipid messengers that bind to the PH domain. Clearly, further study into the role of the PH domain of PDK1 is warranted.

Previous studies have suggested that all PKC subclasses can form complexes with PDK1. For example, PDK1 immunoprecipitated with expressed classical (α and βI), novel (δ, ϵ, and ι), and atypical (ζ and λ) PKCs from 293 cells (*9*). The reason the present PDK1 yeast two-hybrid screen did not pick up any of the classical isoforms of PKC is unclear. Although this may be a consequence of the levels of the proteins in the library, it is also possible that the association in yeast between PDK1 and the classical isoforms of PKC is fundamentally different or may require additional factors.

Most AGC kinases are regulated by phosphorylation on two residues. One is present in the activation loop of the kinase domain, the phosphorylation of which is catalyzed by PDK1. The other resides in the C-terminal tail, the phosphorylation of which is believed either to be under the

control of a putative PDK2 enzyme which has eluded detection or to occur by autophosphorylation. Thus, conventional PKCs, such as PKCβII, autophosphorylate at the PDK2 site by an intramolecular mechanism. Expression studies with kinase inactive mutants suggest that autophosphorylation is the only mechanism responsible for phosphorylating the PDK2 site of conventional PKCs in vivo (*39*). Atypical PKCs, such as PKC-ζ and -λ, and PRK2 lack the PDK2 site and instead possess an acidic residue at this position. However, for a number of other AGC kinases that possess the PDK2 site, such as PKB, evidence suggesting the involvement of a PDK2 activity has been presented. Several PDK2 candidates have recently been proposed, these being ILK (*10*), MK2 (*11*), and the conversion of PDK1 into PDK2 by a small C-terminal fragment of PRK2 termed PIF (*40*, *41*). However, doubt has been cast upon all these being PDK2 enzymes. ILK has been shown to phosphorylate PKB on S473, the PDK2 site, both in vivo and in vitro (*10*). In contrast, the use of ILK mutants suggests that ILK does not possess intrinsic S473 kinase activity but rather functions as an adaptor to recruit a PDK2 enzyme (*14*). MK2, a direct target of p38, is believed also to stimulate PKB S473 phosphorylation. Chemical inhibition of p38 kinase in human neutrophils blocked S473 phosphorylation but not that for T308, the PDK1 site in PKB, in the presence of fMLP (*11*). However, a role for MK2 in intact cells has also been discounted; thus, fibroblasts exhibit IGF-1-dependent PKB activation in the absence of MK2 activation. MK2 activation has also been shown to occur independently of phospholipid production which is necessary for PDK1 function (*15*). It has been reported that, upon binding to the C-terminal 77 amino acids of PRK2, termed PIF, PDK1 is capable of phosphorylating S473 of PKBα (*40*, *41*). However, larger fragments of PRK2 and the close relative PRK1 have the opposite effect. A caspase-3-generated (*22*, *23*) fragment of PRK2 (*16*), its isoform PRK1 (*17*), and the carboxyl terminus of PRK1 (*17*) all inhibited PKB phosphorylation on both T308 and S473 in response to coexpression of an active mutant of PDK1 (*17*) or stimuli such as EGF (*16*). Thus, the mechanism by which PDK1 phosphorylates AGC kinases has remained unclear. Our study shows that the C-terminal region of both PKC-ζ and -δ, though not required for interaction with PDK1, is absolutely required for their phosphorylation by PDK1. This is in agreement with Balendran et al. (*41*), who showed that mutation of residues within the C-terminal tail of PKC-ζ inhibited PDK1 phosphorylation at the PDK1 motif within PKC-ζ. Our study has expanded upon this by showing that the novel PKCs, such as PKC-δ, are regulated by PDK1 in this fashion. It is possible, therefore, that the necessity of the C-terminal tail of PKC-ζ and -δ for their phosphorylation by PDK1 may also be necessary for the phosphorylation of other AGC kinases by PDK1 and that this may be a universal requirement for PDK1 phosphorylation.

We also showed that the caspase-3-generated C-terminal PRK2 fragment (*22*, *23*) reduced the level of autophosphorylation of PDK1 and inhibited PDK1 phosphorylation of PKC-ζ and -δ in vitro. Moreover, generation of the C-terminal PRK2 fragment in vivo resulted in a marked inhibition of PDK1-catalyzed phosphorylation of the activation loop of endogenous PKC-ζ. As autophosphorylation of PDK1 is reported to be associated with catalytically active PDK1 (*41*), these results indicate that an important site of action of the C-terminal PRK2 fragment is at the level of PDK1. This may account for the inhibition of phosphorylation by PDK1 of PKC-ζ and -δ (present work) and PKB (*16*). However, we cannot exclude the possibility that the C-terminal PRK2 fragment also acts at the level of the substrate kinases. The reduction in the level of autophosphorylation of PDK1 by the C-terminal fragment of PRK2 could explain how PDK1, despite being catalytically active in vitro, can be kept inactive in vivo under specific circumstances.

PKB (*43*−*48*) and PKC-ζ (*49*−*54*) have both been implicated in prosurvival signaling and as such have been reported to be necessary for cell survival and the prevention of apoptosis. However, apoptosis often occurs despite the presence of agonists that stimulate the prosurvival pathway. Additionally, the death signaler TNF induces apoptosis despite transiently activating PKB (*55*). This indicates that cells are likely to possess mechanisms that restrain prosurvival signaling to ensure cell death responses.

The C-terminal PRK2 fragment is produced by caspase-3 cleavage of PRK2 during apoptosis. Thus, inhibition at the level of PDK1 and the phosphorylation of critical prosurvival kinases such as PKC-ζ and PKB may provide a general mechanism for the inhibition of prosurvival pathways during apoptosis, leading to the amplification of proapoptotic signaling.

To summarize, we have determined that PDK1 phosphorylation of PKC-ζ and -δ is dependent upon the C-terminal tail of the PKCs. In addition, the C-terminal PRK2 fragment, produced during apoptosis, was found to be a negative regulator of PDK1 and reduced the level of phosphorylation of both PKC-ζ and -δ by PDK1. In conclusion, the C-terminal PRK2 fragment provides a novel mechanism for the inhibition of signaling through PDK1 and phosphorylation of its target kinases. This may provide a route for the suppression of prosurvival signals during apoptosis.

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