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The Carboxyl Terminus of Protein Kinase C Provides a Switch to Regulate Its Interaction with the Phosphoinositide-dependent Kinase, PDK-1*

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The function of protein kinase C family members depends on two tightly coupled phosphorylation mechanisms: phosphorylation of the activation loop by the phosphoinositide-dependent kinase, PDK-1, followed by autophosphorylation at two positions in the COOH terminus, the turn motif, and the hydrophobic motif. Here we address the molecular mechanisms underlying the regulation of protein kinase C βII by PDK-1. Co-immunoprecipitation studies reveal that PDK-1 associates preferentially with its substrate, unphosphorylated protein kinase C, by a direct mechanism. The exposed COOH terminus of protein kinase C provides the primary interaction site for PDK-1, with co-expression of constructs of the carboxyl terminus effectively disrupting the interaction *in vivo*. Disruption of this interaction promotes the autophosphorylation of protein kinase C, suggesting that the binding of PDK-1 to the carboxyl terminus protects it from autophosphorylation. Studies with constructs of the COOH terminus reveal that the intrinsic affinity of PDK-1 for phosphorylated COOH terminus is over an order of magnitude greater than that for unphosphorylated COOH terminus, contrasting with the finding that PDK-1 does not bind phosphorylated protein kinase C effectively. However, effective binding of the phosphorylated species can be induced by the activated conformation of protein kinase C. This suggests that the carboxyl terminus becomes masked following autophosphorylation, a process that can be reversed by the conformational changes accompanying activation. Our data suggest a model in which PDK-1 provides two points of regulation of protein kinase C: 1) phosphorylation of the activation loop, which is regulated by the intrinsic activity of PDK-1, and 2) phosphorylation of the carboxyl terminus, which is regulated by the release of PDK-1 to allow autophosphorylation.

The phosphoinositide-dependent kinase (PDK-1),[1] plays a pivotal role in cellular signaling by regulating the activation state of diverse protein kinases (1). Such protein kinases contain a segment near the entrance to the active site that must be phosphorylated in order to correctly align residues for catalysis. This segment, the activation loop, typically blocks the active site in the inactive conformation and moves out following phosphorylation (2). Although some kinases (*e.g.* protein kinase A) are able to self-activate by autophosphorylating at the activation loop (3), a large number of kinases depend on an upstream activation loop kinase for phosphorylation. PDK-1 was originally discovered as the activation loop kinase for Akt/protein kinase B (4). Following on the heels of this discovery was the finding that PDK-1 is also the upstream kinase for p70 S6 kinase (5, 6) and both atypical (7, 8) and conventional (9) isoforms of protein kinase C. The list of PDK-1 substrates continues to grow, placing PDK-1 in the center of a multitude of signaling pathways, from protein synthesis to cell growth and survival (10, 11).

Phosphorylation by PDK-1 is the first of three ordered phosphorylations in the maturation of protein kinase C (9). These phosphorylations are required to stabilize the catalytically competent conformation of the enzyme and to localize the mature enzyme to the cytosol (12–15). It is the phosphorylated species of protein kinase C that transduces the myriad of signals resulting in generation of diacylglycerol (16, 17). Mature (*i.e.* phosphorylated) species of conventional (α, βI, βII, and γ) and novel isozymes (δ, ϵ, θ, and η) typically localize to the cytosol but translocate to the membrane upon generation of diacylglycerol. The membrane interaction is mediated by two membrane-targeting modules, the C1 domain which binds diacylglycerol and phosphatidylserine, and the C2 domain which, in conventional isozymes, binds anionic phospholipids in a Ca^{2+}-dependent manner (18). Engagement of these domains on the membrane provides the energy to release an autoinhibitory pseudosubstrate sequence from the substrate-binding cavity, which results in activation of the enzyme. Extensive biochemical, biophysical, and cell biological studies over the past two decades have led to a strong understanding of how mature protein kinase C is regulated allosterically by lipid second messengers. However, how the maturation of protein kinase C is regulated remains to be resolved.

In addition to the activation loop, mature protein kinase C is phosphorylated at two conserved positions in the carboxyl terminus (19): the turn motif (Thr^{641} in protein kinase C βII) so named because it corresponds to a phosphorylation site in protein kinase A localized at the apex of a turn, and the hydrophobic motif (Ser^{660} in protein kinase C βII) which comprises a Ser flanked by bulky hydrophobic residues. The phosphorylation at the activation loop that is catalyzed by PDK-1 triggers the intramolecular autophosphorylation of these two positions (20). Because the first step mediated by PDK-1 is the rate-limiting step, understanding how it is regulated is key to un-

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[1] The abbreviations used are: PDK-1, phosphoinositide-dependent kinase 1; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PCR, polymerase chain reaction; GST, glutathione *S*-transferase.

derstanding the cellular controls of protein kinase C function.

A number of studies suggest that PDK-1 interacts with significant affinity with its kinase substrates. For example, protein kinase C isozymes and p70 S6 kinases are present in immune complexes of PDK-1 (7–9). Thus, understanding how PDK-1 recognizes its substrates may provide a first step in understanding how this master kinase regulates the function of its substrate kinases.

This study addresses how PDK-1 recognizes and regulates protein kinase C βII *in vivo*. By examining the ability of PDK-1 to complex with various constructs of protein kinase C and its isolated domains *in vivo*, we show that PDK-1 interacts primarily with determinants residing in the COOH-terminal hydrophobic phosphorylation motif of protein kinase C. The binding of PDK-1 inhibits the intramolecular autophosphorylation required for the maturation of protein kinase C, with release of PDK-1 exposing the COOH terminus for autophosphorylation. Thus, PDK-1 regulates both phosphorylation switches of protein kinase C: the activation loop by direct phosphorylation and the COOH-terminal sites by release from protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—All reagents were obtained from general sources unless otherwise stated. The large T-antigen transformed human embryonic kidney cells (tsA201) were the generous gift of Dr. Marlene Hosey (Northwestern University). The cDNA of rat PKC βII was a gift of Dr. Daniel E. Koshland, Jr. (University of California, Berkeley). A polyclonal antibody against the COOH terminus of PKC βII was purchased from Santa Cruz Biotechnology. A phospho-specific antibody (P500) that specifically recognizes the phosphorylated activation loop of protein kinase C isozymes was generated and characterized as described previously (9, 21). A phospho-specific antibody (labeled P660; referred to as Pan-phosphorylated PKC by vendor) against the phosphorylated Ser^{660} in the COOH terminus of PKC βII was obtained from New England Biolabs.

Plasmid Constructs—A mammalian expression construct encoding the NH_2-terminal Myc-tagged PDK-1 in pcDNA3 has been described previously (7). The cDNAs encoding the wild-type and several phosphorylation site mutants of PKC βII, including PKCβII-T447A/T448A/T500A(T500A),[2] PKCβII-T634A/T638A/T641A (T641A),[3] PKCβII-S660A, PKCβII-S660E, PKCβII-T500E/T641E/S660E (E3), and PKCβII-F656A/F659A (FA2), were subcloned into the pcDNA3 vector for expression in mammalian cells (9, 14, 15, 20, 22). The C1 domain (residues 1–156), the C2 domain (residues 157–296), the catalytic domain (termed CD, resides 296–673), and the COOH terminus (termed CT, residues 628–673) of protein kinase C βII were expressed as glutathione *S*-transferase (GST) fusion proteins in mammalian cells following PCR amplification of the relevant sequences using pcDNA3PKCβII as the template. Specifically, the primers used for the PCR amplification introduced a *Bam*HI site and a *Not*I site at the 5′ and 3′ ends, respectively. The PCR products were subcloned into the pEBG vector digested with *Bam*HI and *Not*I. For expression of the PKC βII COOH terminus with mutations S660A, S660E, T641E/S660E (E2), or FA2, the same PCR amplification procedures were performed except that the templates containing the corresponding mutations were used. The resulting expression constructs encoded the following fusion proteins including GST-CT, GST-CT/S660A, GST-CT/S660E, GST-CT/E2, GST-CT/FA2, GST-C1, GST-C2, and GST-CD. As a control, the COOH terminus of PRK2 (residues 908–984; previously termed PIF (23)) was PCR amplified from PRK2 (generous gift of Margaret Chou, University of Pennsylvania) and subcloned into the pEBG vector for expression as a GST fusion protein in mammalian cells.

Cell Transfection—TsA201 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Transient transfection of tsA201 cells was carried out using Effectene transfection reagents (Qiagen). The specific transfection procedures were performed according to the protocol suggested by Qiagen. Combinations of the different expression plasmids were used as stated, and 1 μg of each DNA construct was generally included in the transfection.

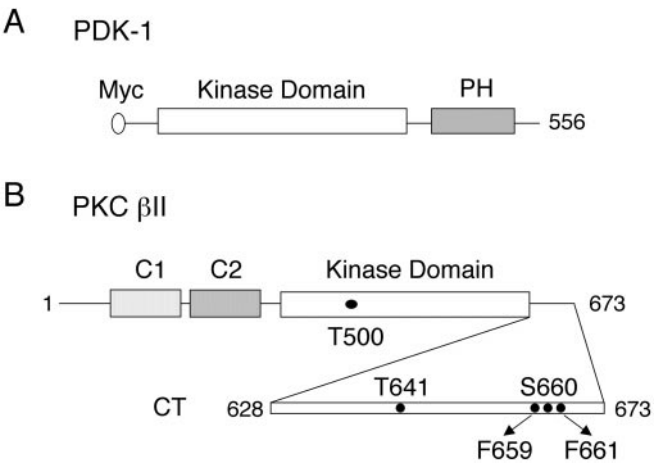

FIG. 1. **Schematic representation of PDK-1 and protein kinase C βII constructs used in this study.** *A,* wild-type PDK-1, containing an NH_2-terminal kinase domain and a COOH-terminal PH domain, was NH_2 terminally tagged with a Myc epitope. *B,* primary structure of wild-type PKC βII showing the C1 and C2 domains in the NH_2-terminal regulatory moiety and the kinase domain in the COOH-terminal moiety. Thr^{500} is in the activation loop of PKC βII and phosphorylated by PDK-1. Also indicated are the COOH-terminal constructs (CT) of protein kinase C βII comprising residues 628 to 673. The autophosphorylation sites, Thr^{641} and Ser^{660}, in the COOH terminus of PKC βII are indicated, as are the two hydrophobic residues Phe^{659} and Phe^{661} flanking Ser^{660}. The *numbers* at the COOH-terminal end of each construct indicate the last amino acid residue of the protein.

GST Fusion Protein Pull-down Assay—To examine the interaction between PDK-1 and different domains of protein kinase C βII *in vivo*, tsA201 cells were transiently transfected with Myc-tagged PDK-1 and the wild-type or the mutant GST-CTs. Approximately 40 h post-transfection, the transfected cells were lysed in buffer A (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, 300 μM phenylmethylsulfonyl fluoride, and 300 nM okadaic acid). The lysate was cleared by centrifugation at 13,000 rpm, 5 min, 22 °C and the resulting supernatant is referred to as the detergent-solubilized cell lysate. Ten percent of the total cell lysate was kept in SDS sample buffer for further analysis, and the remaining cell lysate was incubated with glutathione-Sepharose at 4 °C for overnight. After washing twice in buffer A and twice in buffer B (buffer A plus 300 mM NaCl), the glutathione-Sepharose bound proteins were analyzed using SDS-PAGE and immunoblotting.

Immunoprecipitation—TsA201 cells were transiently transfected with different combinations of protein kinase C βII and Myc-PDK-1. Approximately 40 h post-transfection, the cells were lysed in buffer A. Ten percent of the total detergent-solubilized cell lysates was quenched in SDS sample buffer for further analysis, and the remaining detergent-solubilized cell lysate was incubated with an anti-Myc monoclonal antibody and protein A/G-agarose (Santa Cruz Biotechnology) at 4 °C overnight. To examine the direct interaction between PDK-1 and protein kinase C βII, Sf21 insect cells were infected with baculovirus encoding 6His-tagged PDK-1 or protein kinase C βII. The 6His-tagged PDK-1 and protein kinase C βII were purified as described previously (24). The catalytic domain of protein kinase C was generated by incubation of pure protein kinase C βII (1.5 μg ml^{-1}) with trypsin (1.2 units ml^{-1}) for 10 min at 30 °C, in the presence of 1 mM Ca^{2+}, as previously described (25). The purified proteins were combined in buffer A and incubated with an anti-PDK-1 antibody (Upstate Biotechnology) in the presence or absence of protamine sulfate (50 μg ml^{-1}) at 4 °C for overnight. The immunoprecipitates were washed twice in buffer A and twice in buffer B. The proteins in the immunoprecipitates were separated using SDS-PAGE and analyzed using immunoblotting.

RESULTS

We have previously reported that PDK-1 and protein kinase C βII associate *in vivo*, as assessed by co-immunoprecipitation studies in HEK cells co-transfected with the cDNA for each kinase (9). To further examine the mechanism of this interac-

[2] All three potential phosphorylation sites around the PDK-1 site were mutated to prevent compensating phosphorylations resulting from mutation of Thr^{500} to Ala.

[3] Surrounding phosphorylation sites were mutated to prevent compensating phosphorylations that occur when Thr^{641} is mutated to Ala (15).

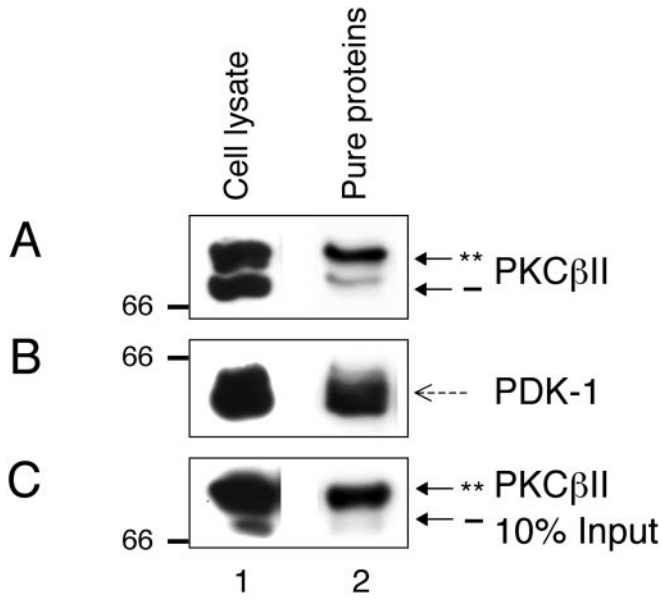

FIG. 2. **Direct interaction between PDK-1 and protein kinase C βII.** TsA201 cells were transiently transfected with PKC βII and Myc-tagged PDK-1. Detergent-solubilized cell lysates were immunoprecipitated with the anti-Myc antibody coupled to protein A/G-agarose to precipitate PDK-1 (*lanes 1*). Alternatively, 6His-tagged PDK-1 and protein kinase C βII were first purified from recombinant baculovirus infected Sf21 cells, and the mixture of two proteins were immunoprecipitated with an anti-PDK-1 antibody coupled to protein A/G-agarose (*lane 2*). The immunoprecipitates were analyzed using SDS-PAGE and immunoblotting. *A,* co-immunoprecipitated PKC βII was detected using an anti-PKC βII antibody that labels both the unphosphorylated and phosphorylated protein kinase C. *B,* PDK-1 in the immune complexes was detected using the anti-PDK-1 antibody. *C,* 10% of the input proteins were analyzed for total protein expression of PKC βII using the anti-PKC βII antibody. The unphosphorylated and phosphorylated species of PKC βII are indicated by a *dash* (−) and *double asterisks* (**), respectively. The *dashed line arrow* indicates PDK-1. The 66-kDa molecular mass marker is indicated on the *left*.

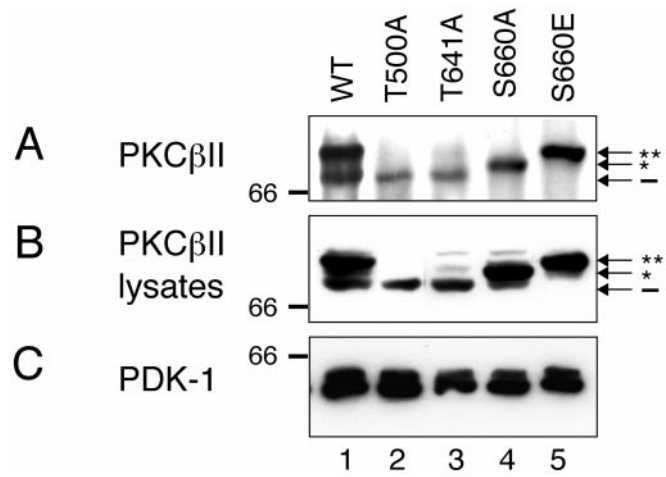

FIG. 3. **Co-immunoprecipitation of PDK-1 with phosphorylation site mutants of protein kinase C βII.** TsA201 cells were transiently transfected with the Myc-tagged PDK-1 and one of the following PKC βII constructs (*lanes 1–5*, respectively): PKC βII wild-type, T500A, T641A, S660A, and S660E mutants. Detergent-solubilized cell lysates were immunoprecipitated with the anti-Myc antibody coupled to protein A/G-agarose to precipitate PDK-1. The immunoprecipitates were analyzed for the presence of PKC βII using SDS-PAGE and immunoblotting. *A,* co-immunoprecipitated wild-type and mutant protein kinase C βII were detected using the anti-PKCβII antibody. The *dash* indicates the position of the fastest migrating (unphosphorylated) species of PKC βII, *asterisk* (*) indicates the position of protein kinase C with negative charge at only one of the two COOH-terminal phosphorylation sites (Thr^{641} or Ser^{660}), and *double asterisk* (**) indicates position of fully phosphorylated protein kinase C. *B,* 10% of whole cell lysates were immunoblotted using the anti-PKCβII antibody to show the expression of the wild-type and mutant protein kinase C βII. *C,* PDK-1 in the immunoprecipitates was detected using the anti-Myc antibody. The 66-kDa molecular mass marker is indicated on the *left*.

tion and how it is regulated, we identified the determinants of protein kinase C that mediate the interactions and addressed how the interaction is affected by the phosphorylation state and activation state of protein kinase C. Fig. 1 shows the constructs of PDK-1 and protein kinase C used in this study.

PDK-1 Preferentially Binds Unphosphorylated Protein Kinase C—Myc-tagged PDK-1 and wild-type protein kinase C βII were co-expressed in tsA201 cells and the detergent-soluble supernatant (containing ~90% of the expressed constructs) was subjected to immunoprecipitation using the anti-Myc antibody. Fig. 2, *lane 1*, shows that a significant fraction of the protein kinase C co-immunoprecipiated with PDK-1, consistent with our previous report (9). Two species of protein kinase C were apparent in the immunoprecipitates (*panel A*): a slower migrating band (indicated by *double asterisk*) which represents fully phosphorylated protein kinase C (see Ref. 19) and was labeled by the phosphoactivation loop antibody (P500, not shown), and a faster migrating species (labeled with a *dash*) which represents unphosphorylated protein kinase C and was not labeled by the P500 antibody (not shown). The dephosphorylated species in the immune complex was significantly enriched relative to the amount in the total cell lysate: densitometric analysis of six independent experiments revealed a 2.0 ± 0.7-fold enrichment of the dephosphorylated species relative to the phosphorylated species in the immune complexes compared with the ratio of these species in the total lysate. Thus, PDK-1 selectively bound unphosphorylated protein kinase C.

To probe whether the interaction of protein kinase C with PDK-1 was direct or mediated by a scaffold protein, we examined the interaction of pure PDK-1 with pure protein kinase C. Both proteins were expressed in baculovirus-infected insect cells and purified to apparent homogeneity. Fig. 2, *lane 2*, shows that protein kinase C βII was present in immune complexes with PDK-1 (*panel A*). As observed using cell lysates, the immune complex was enriched in the unphosphorylated species (*dash*) of protein kinase C: this species was barely apparent in the starting material (*panel C*) but readily apparent in the immune complex (*panel A*). The efficiency of immunoprecipitation of protein kinase C by PDK-1 in cell lysates or *in vivo* was comparable, suggesting that direct interaction of the two proteins was the primary mechanism for the interaction observed *in vivo*.

Mutations in the Carboxyl-terminal Hydrophobic Motif of Protein Kinase CβII Disrupt the PDK-1/PKCβII Interaction in Vivo—The results described above indicate that PDK-1 preferentially associates with its substrate, unphosphorylated protein kinase C βII, compared with the mature, fully phosphorylated protein kinase C βII. This latter species is phosphorylated at three positions: Thr^{500} on the activation loop, and Thr^{641} and Ser^{660} on the carboxyl terminus. To test whether the phosphorylation at these positions modulates association of protein kinase C with PDK-1, we examined the ability of constructs mutated at each position for the ability to form an immune complex with PDK-1. The detergent-soluble cell lysates were immunoprecipitated with the anti-Myc antibody to precipitate PDK-1 and the immunoprecipitates probed for associated protein kinase C. Fig. 3*C* shows that the amount of PDK-1 was similar among all the immunoprecipitates.

Fig. 3*B* shows a Western blot of the wild-type and phosphorylation site mutants of protein kinase C βII expressed in tsA201 cells. Wild-type protein kinase C (*lane 1*) migrated as a

doublet, similar to the results in Fig. 2. In contrast, both the T500A[2] and T641[3] mutants co-migrated with unphosphorylated protein kinase C (Fig. 3*B*, position indicated by *dash*). We have previously shown that mutation of Thr^{500} to Ala or Thr^{641} to Ala abolishes kinase activity and thus results in expression of quantitatively dephosphorylated protein kinase C (15, 22, 26).[4] Mutation of Ser^{660} to Ala resulted in the appearance of a major intermediate migrating band (*lane 4*, position marked with *asterisk*) and a minor slower migrating band (*lane 4*, position indicated by *dash*). This is consistent with previous studies showing that mutation of Ser^{660} to Ala has only modest effects on the activity of protein kinase C so that the majority of the S660A that partitions in the detergent-soluble fraction is phosphorylated at Thr^{500} and Thr^{641}: this phosphorylated species migrates at an intermediate mobility. Replacement of Ser^{660} with Glu results in unimpaired phosphorylation of protein kinase C so that the S660E mutant is a good mimic of wild-type enzyme and co-migrates with wild-type enzyme (Fig. 3*B, lane 5*).

Fig. 3*A* shows that the T500A and T641A constructs were effectively co-immunoprecipitated with PDK-1 (*lanes 2* and *3*), but the T500A mutant has slightly reduced efficiency compared with wild-type enzyme. Quantitation of several independent experiments revealed that 0.5 ± 0.1 (n = 4) and 0.8 ± 0.1 (n = 3) times as much T500A and T641A, respectively, were present in immune complexes compared with wild-type unphosphorylated protein kinase C (*lower band* marked by *dash*). The slightly decreased recognition of protein with Ala at position 500 suggests that the hydroxyl at the phosphoacceptor position may influence the recognition of protein kinase C by PDK-1.

To test whether negative charge at position 660 regulated the interaction of mature protein kinase C with PDK-1, we examined the ability of PDK-1 to complex with the S660A and S660E mutants *in vivo*. Fig. 3 reveals the presence of intermediate migrating species (marked by *one asterisk*), the S660A mutant, in the immune complexes with PDK-1 (Fig. 3*A, lane 4*). This species represents protein kinase C phosphorylated at the activation loop and Thr^{641}. Quantitative analysis of three independent experiments revealed the fraction of S660A co-immunoprecipitated from the lysate with PDK-1 was 0.4 ± 0.2-fold lower than the fraction of wild-type protein kinase C that was co-immunoprecipitated. These data suggest that replacement of Ser^{660} with Ala results in a 2.5-fold reduction in the affinity of mature protein kinase C for PDK-1. PDK-1 was also able to effectively co-immunoprecipitate the S660E mutant (Fig. 3*A, lane 5*). As described previously, the S660E mutant expressed primarily as the slowest migrating species co-migrating with the fully phosphorylated wild-type protein kinase C βII (Fig. 3*B, lane 5, double asterisk*). The fraction of S660E that co-immunoprecipitated with PDK-1 was similar to the fraction of fully phosphorylated protein kinase C βII that complexed with PDK-1 (Fig. 3*A*, compare *lanes 1* and *5*; analysis of three independent experiments revealed that the ratio of S660E to wild-type protein kinase C in immune complexes was 1.0 ± 0.3). Thus, mature protein kinase C with a Glu or phosphoserine at position 660 interacted similarly with PDK-1, whereas mature protein kinase C with a Ala at position 660 interacted less strongly with PDK-1 than protein with phospho-Ser at that position.

Interaction of Different Domains of Protein Kinase C CβII with PDK-1 in Vivo—In vivo co-precipitation assays reveal that PDK-1 preferentially recognizes the unphosphorylated conformation of protein kinase C. To elucidate the contribution of individual domains in this recognition, we tested the ability of GST fusion proteins encoding the C1, C2, and catalytic domains of protein kinase C βII to associate with PDK-1 or to disrupt the interaction of PDK-1 with full-length protein kinase C βII *in vivo*. In addition, we focused on a series of constructs of the carboxyl-terminal 46 residues to explore the role of the hydrophobic phosphorylation motif in the PDK-1/protein kinase C interaction. These constructs included GST-CT, GST-CT/S660A, GST-CT/S660E, GST-CT/E2, and GST-CT/FA2. We also examined the interaction with PIF, a COOH-terminal construct of PRK-2 previously shown to bind with high affinity to PDK-1 (23). TsA201 cells were transiently co-transfected with PDK-1 and the GST fusion protein constructs and the interaction with PDK-1 was assessed by GST pull-down experiments performed using the detergent-solubilized fraction of cells. The total protein expression level of PDK-1 was comparable in all the transfections (Fig. 4*D*).

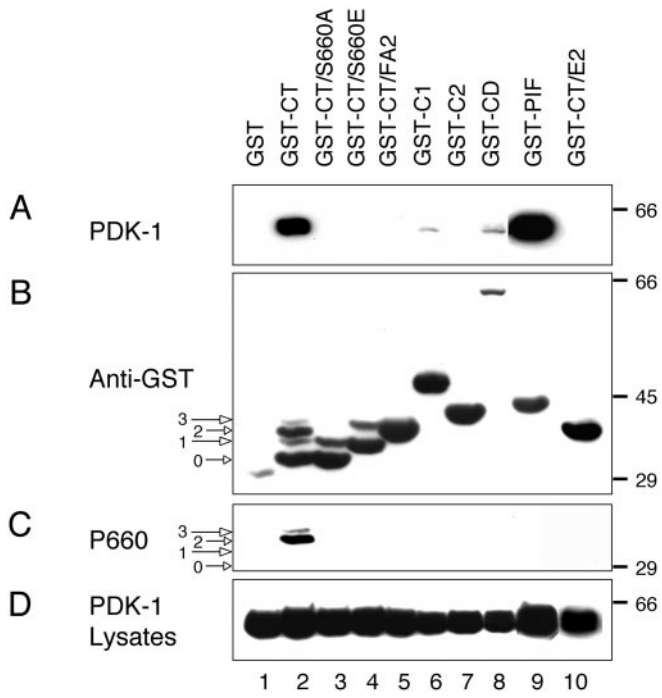

FIG. 4. **Association of PDK-1 with different domains of protein kinase C βII.** The GST-tagged fusion proteins encoding different domains of protein kinase C βII, with or without point mutations, were co-expressed with the Myc-tagged PDK-1 in tsA201 cells: GST-CT (the wild-type COOH terminus), GST-CT/S660A, GST-CT/S660E, GST-CT/FA2, GST-C1, GST-C2, and GST-CD (catalytic domain). The GST vector and GST-tagged PIF were included as the negative and the positive controls, respectively. Detergent-solubilized cell lysates were incubated with glutathione-Sepharose to precipitate the GST fusion proteins. The proteins that bound to the glutathione beads were analyzed using SDS-PAGE and immunoblotting. *A,* the presence of PDK-1 in the glutathione-Sepharose precipitates was detected using the anti-Myc antibody. *B,* the GST fusion proteins precipitated by the glutathione-Sepharose were detected using the anti-GST antibody. Note that the wild-type GST-CT fusion proteins migrated as multiple bands on the blot. These bands are marked by *arrows* and labeled as *bands 0, 1, 2* and *3*. *C,* the blot of glutathione-Sepharose bound proteins was probed with a phospho-specific P660 antibody. *D,* the expression of PDK-1 in 10% of total cell lysates was analyzed using the anti-Myc antibody. The 66,000, 45,000, and 29,000 molecular weight markers are indicated on the *right*.

Fig. 4*B* reveals that all constructs of the COOH terminus, including PIF, were expressed in tsA201 cells and effectively precipitated by glutathione-Sepharose as judged by staining with anti-GST antibodies. Of these, the GST fusions of the wild-type COOH terminus and of PIF (Fig. 4*A, lanes 2* and *9*) were the most effective at co-precipitating PDK-1. To obtain relatively balanced signals on the immunoblot, PDK-1 detected

[4] The T641A mutant is a substrate for PDK-1, however, it is highly phosphatase sensitive and not phosphorylated *in vivo* under our cell culture conditions (15).

in the GST-PIF lane represented only 30% of the total protein in the glutathione-Sepharose precipitates. Densitometric analysis of data from three independent experiments revealed that PDK-1 bound PIF 5–10 times better than the COOH terminus of protein kinase C βII. In contrast to the wild-type COOH-terminal constructs, neither the S660A, S660E, nor T641E/S660E constructs of the COOH terminus showed detectable interaction with PDK-1 (Fig. 4*A, lanes 3, 4,* and *10*). A construct in which the two Phe flanking Ser^{660} were mutated to Ala, GST-F659A/F661A, did not complex with PDK-1 (Fig. 4*A, lane 5*). A construct of the catalytic domain comprising the kinase core and the COOH terminus (residues 296–673) was poorly expressed in the tsA201 cells yet was co-complexed with PDK-1 (Fig. 4*B, lanes 8*). The GST fusion constructs of the isolated C1 and C2 domains expressed well in tsA201 cells and GST pull-down experiments revealed trace binding of PDK-1 to the C1 domain but not to the C2 domain (*lanes 6* and *7*). These data suggest that the kinase domain, and in particular the COOH terminus, provide the primary determinants in the interaction of protein kinase C with PDK-1, with some participation of the C1 domain.

Fig. 4*B* reveals that the COOH-terminal fusion protein migrated as multiple bands on SDS-PAGE (*lane 2*), suggesting potential modification by phosphorylation. Expression of the GST-carboxyl-terminal construct resulted in the appearance of 4 bands: a major fastest migrating band and three minor slower migrating bands. These 4 bands are labeled *0, 1, 2,* and *3* on Fig. 4*B*. Both the GST-CT/S660A and S660E constructs migrated as 2 bands, with the S660A construct co-migrating with the two lower wild-type bands (bands 0 and 1) and the S660E construct co-migrating with the two upper wild-type bands (bands 1 and 3). In addition, a construct in which both Thr^{641} and Ser^{660} were mutated to Glu (GST-CT/E2) migrated as a single band, whose mobility was the same as that of the slower migrating form of the wild-type construct (band 2). This suggests that a fraction of the COOH-terminal construct becomes phosphorylated at position 660 and/or 641 *in vivo*. Western blot analysis using an antibody that specifically recognizes phosphorylated Ser^{660} (P660) revealed that the two slowest upper migrating band (bands 2 and 3) of the wild-type COOH terminus are, indeed, phosphorylated on Ser^{660} (Fig. 4*C, lane 2*). This antibody did not label the GST-CT/S660A, GST-CT/S660E, or a double mutant, GST-CT/E2 (Fig. 4*C, lanes 3, 4,* and *10*). These data reveal that a minor fraction (typically 10–20%) of the wild-type COOH terminus is phosphorylated on Ser^{660}, and an even smaller fraction on Ser^{660} and one other position, possibly Thr^{641}. The wild-type GST-CT is effectively phosphorylated *in vitro* at Ser^{660} by protein kinase C suggesting that the weak phosphorylation of this construct observed *in vivo* may be catalyzed by endogenous protein kinase C (data not shown).

Disruption of PDK-1/PKC Interaction by Co-expression of the COOH-terminal Fusion Proteins of PKC βII—To further test the hypothesis that the COOH terminus of protein kinase C is a major determinant in the interaction of PDK-1 with protein kinase C, we tested the ability of the various constructs of the COOH terminus, including PIF, to disrupt the interaction of PDK-1 with protein kinase C βII *in vivo*. TsA201 cells were co-transfected with PDK-1 and protein kinase C, and either GST or the GST-COOH-terminal constructs. PDK-1 was then immunoprecipitated from the detergent-solubilized fraction and the amount of co-precipitated protein kinase C was determined by Western blot analysis. Fig. 5*A* shows that protein kinase C (*panel III*) was expressed to comparable levels in all transfections, and the amount of PDK-1 in all the immunoprecipitates was similar (*panel II*). Furthermore, the protein kinase C in the detergent-solubilized fraction migrated as a major slower migrating species under all transfection conditions (*panel III; double asterisk*), revealing fully phosphorylated mature form. Immunoprecipitation of PDK-1 from cells co-transfected with protein kinase C and GST resulted in significant co-immunoprecipitation of protein kinase C (*panel I, lane 1*). In contrast, co-expression of GST-CT reduced the amount of protein kinase C βII complexed with PDK-1 to barely detectable levels (*panel I, lane 2*). Similarly, co-expression of PIF caused a marked reduction in the amount of protein kinase C associated with PDK-1 (*panel I, lane 6*). In contrast, the GST-CT/S660A, GST-CT/S660E, and GST-CT/FA2 constructs had no significant effect on the PDK-1/protein kinase C interaction (*panel I, lanes 3–5*).

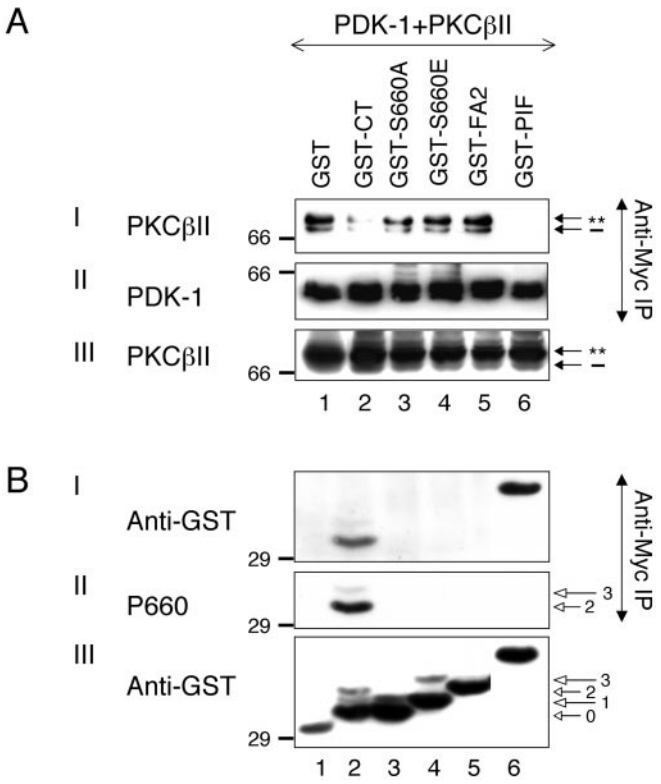

FIG. 5. **Disruption of the PDK-1/protein kinase C βII interaction by co-expression of GST-tagged COOH-terminal fusion proteins of protein kinase C βII.** TsA201 cells were transiently transfected with Myc-PDK-1 and protein kinase C βII together with one of the following GST fusion protein constructs: GST vector, GST-CT, GST-CT/S660A, GST-CT/S660E, GST-CT/FA2, and GST-PIF (*lanes 1–6*, respectively). PDK-1 was immunoprecipitated from detergent-solubilized cell lysates with the anti-Myc antibody coupled to protein A/G-agarose. The immunoprecipitates were analyzed using SDS-PAGE and immunoblotting. *A,* co-immunoprecipitated PKC βII was detected with the anti-PKC βII antibody (*panel I*); PDK-1 in the immunoprecipitates was detected using the anti-Myc antibody (*panel II*). The amount of protein kinase C βII in 10% of the cell lysates was analyzed using the anti-PKC βII antibody (*panel III*). *B,* Western blot of immunoprecipitates from *A* probed with the anti-GST (*panel I*) and the P660 (*panel II*) antibodies. The amount of each GST fusion protein present in 10% of total cell lysates was detected using the anti-GST antibody (*panel III*). The wild-type GST-CT fusion proteins migrated as multiple bands on the blot. These bands are marked by *arrows* and labeled as *bands 0, 1, 2,* and *3*.

Given the unexpected finding that the wild-type COOH-terminal fragment was extremely effective in disrupting the interaction of PDK-1 with protein kinase C *in vivo*, but that neither the S660A nor S660E constructs interfered with the interaction, we hypothesized that the relevant species involved in disrupting the interaction was the phosphorylated COOH-terminal fragment. To test this, we asked whether the phosphorylated wild-type COOH-terminal construct was selectively

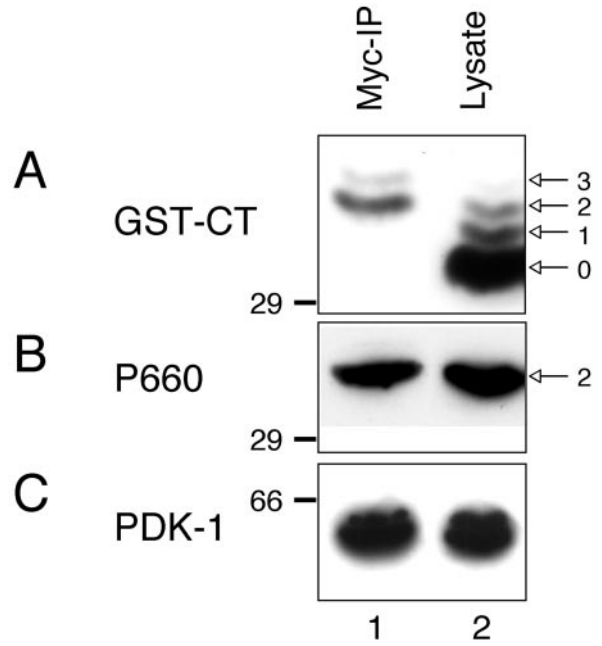

FIG. 6. **PDK-1 preferentially binds to the phosphorylated COOH terminus of protein kinase C βII.** TsA201 cells were transiently transfected with Myc-tagged PDK-1 and the wild-type GST-CT. Detergent-solubilized cell lysates were incubated with the anti-Myc antibody and immunoprecipitates were analyzed using SDS-PAGE and immunoblotting. The GST-CT fusion proteins in the immunoprecipitate (*lane 1*) and the total protein expression in the cell lysates (*lane 2*) were detected by the anti-PKC βII antibody (*A*) or the P660 antibody (*B*). Note that the GST-CT fusion proteins in the lysates migrated as 4 bands (labeled as *bands 0–3*) as detected by the anti-PKC βII antibody, but only the two slower migrating species were in the immunoprecipitate. *C,* the amount of PDK-1 in the immunoprecipitate (*lane 1*) and the amount present in 10% of cell lysates (*lane 2*) was detected using the anti-Myc antibody.

co-immunoprecipitated with PDK-1 in the experiments described in Fig. 5*A*. Fig. 5*B* shows that only two constructs were present in immune complexes of PDK-1 as detected by the anti-GST antibody: the wild-type COOH terminus and PIF (*panel I, lanes 2* and *6*). Importantly, the species of COOH-terminal construct in the immune complex was phosphorylated at position Ser^{660} as judged by labeling with the P660 antibody and its co-migration with the slower migrating species of the COOH-terminal construct (Fig. 5*B, panel II*). The faster migrating species were not present, revealing that phosphorylation of Ser^{660} rendered the COOH terminus effective in disrupting the interaction of PDK-1 with protein kinase C. The full-length protein kinase C and the total COOH-terminal fragment expressed to comparable levels in cell lysates (data not shown), with the fraction of phosphorylated COOH-terminal construct representing only about 10% of the total construct (see Figs. 4*B* and 5*B*). Thus, the ratio of protein kinase C to phosphorylated COOH-terminal construct was about 10:1. The ability of the Ser^{660}-phosphorylated construct to almost quantitatively disrupt the PDK-1/protein kinase C interaction, despite the 10-fold excess of protein kinase C, suggests that it has a significantly higher affinity for PDK-1 than the full-length protein kinase C.

To further test the hypothesis that Ser^{660} phosphorylated GST-CT binds PDK-1 with higher affinity compared with the unphosphorylated species, we performed a co-immunoprecipitation experiment using tsA201 cells co-expressing PDK-1 and GST-CT (Fig. 6). PDK-1 was immunoprecipitated using anti-Myc antibodies from the detergent-solubilized cell lysates and the phosphorylation state of the COOH-terminal construct associated with the immunoprecipitate was examined. The expression of GST-CT and PDK-1 was detected in the cell lysate (Fig. 6, *lane 2*). Consistent with the results shown above, GST-CT expressed as multiple bands in the transfected cells. However, only the species of GST-CT phosphorylated on Ser^{660} was co-immunoprecipitated with PDK-1 as assessed by its labeling with the P660 antibody (Fig. 6*B*) and retarded electrophoretic mobility (Fig. 6*A, lane 1*). These data further confirm that PDK-1 selectively bound the Ser^{660}-phosphorylated COOH-terminal fusion protein of PKC βII.

Accessibility of the COOH terminus of Protein Kinase C Is Conformationally Sensitive—The above data reveal that PDK-1 selectively binds unphosphorylated protein kinase C compared with phosphorylated protein kinase C (Fig. 2). Yet, studies with the isolated COOH terminus show that the intrinsic affinity of PDK-1 is much greater for COOH-terminal constructs that are phosphorylated at Ser^{660} (Figs. 5 and 6). Because protein kinase C undergoes global conformational changes following phosphorylation, we addressed the possibility that the COOH terminus of protein kinase C becomes masked following the maturation of the enzyme. We have previously shown that the pseudosubstrate of protein kinase C is exposed in the unphosphorylated conformation of protein kinase C, masked in the phosphorylated but inactive conformation, and then exposed again following activation (24, 26). This led us to explore whether inducing the "open" conformation of activated protein kinase C might expose the COOH terminus and promote more efficient binding of PDK-1. Protein kinase C was activated by incubation with protamine sulfate, a cofactor-independent substrate that promotes the activating conformation of protein kinase C (27). Fig. 7*A* shows that protamine sulfate resulted in a significant increase (~3-fold) in the amount of purified protein kinase C βII present in immune complexes with purified PDK-1. This effect was specific for full-length protein kinase C: the proteolytically generated kinase domain of protein kinase C interacted equally well with PDK-1 in the presence or absence of protamine sulfate (Fig. 7*B*). A similar increase in binding of protein kinase C to PDK-1 was observed following activation by PMA (data not shown). These data suggest that the open conformation induced by binding protamine increases the binding of PDK-1, presumably by unmasking the COOH-terminal docking site. Since the kinase domain alone is not masked by the pseudosubstrate or interactions with other determinants in the regulatory domain, protamine would not be expected to alter the conformation or accessibility of the COOH terminus of the kinase domain.

Co-expression of the Carboxyl-terminal Fragment of Protein Kinase C Increased Autophosphorylation of the Full-length Protein Kinase C—To examine the effect of co-expression of the COOH terminus of protein kinase C on the autophosphorylation of the full-length protein kinase C, tsA201 cells were transfected with PKC βII and PDK-1 in the presence or absence of the COOH-terminal fragment. In the experiment shown in Fig. 8, protein kinase C βII expressed as a doublet in cell lysates, with the faster migrating species (marked by a *dash*) representing unphosphorylated protein kinase C and the slower migrating species (marked by *double asterisk*) representing fully phosphorylated enzyme (Fig. 8*B*). Consistent with the data in Fig. 5, expression of the COOH-terminal fragment abolished the stable interaction of PDK-1 with protein kinase C (Fig. 8*A, lane 3*). When the COOH-terminal fragment was co-expressed, the species of protein kinase C present in the lysate was shifted exclusively to the slower migrating form (Fig. 8*B*, compare *lane 3* with *lanes 1* and *2*). Thus, the COOH-terminal fragment promoted the maturation of protein kinase C. These data suggest that release of PDK-1 from protein

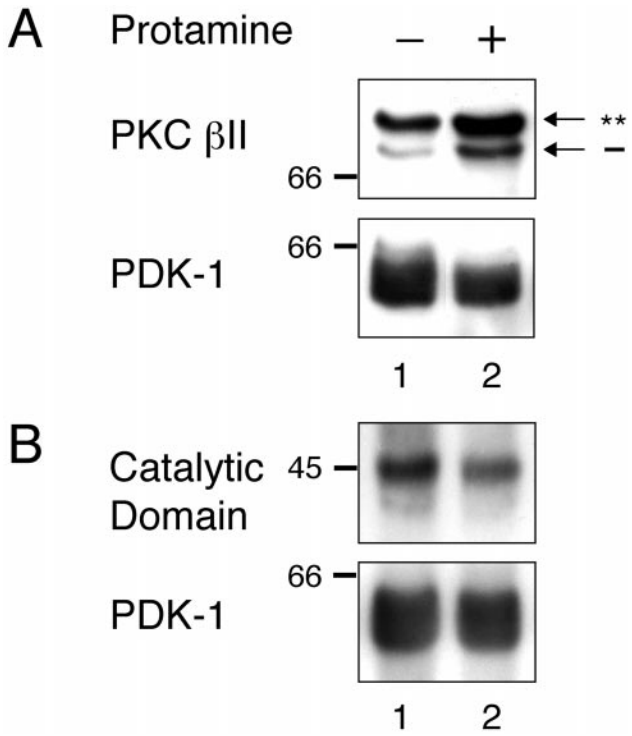

FIG. 7. **Conformational changes of protein kinase C βII increase binding of PDK-1 with the phosphorylated species of protein kinase C.** *A,* the 6His-tagged PDK-1 and protein kinase βII were purified from recombinant baculovirus-infected Sf21 cells, and the mixture of two proteins were immunoprecipitated with the anti-PDK-1 antibody coupled to protein A/G-agarose with or without co-incubation with 50 μg ml^{-1} of protamine sulfate. *B,* as a control, the purified full-length protein kinase βII was first treated with trypsin to generate the catalytic domain as the proteolytic product. The 6His-tagged PDK-1 and the catalytic domain of protein kinase C βII were co-immunoprecipitated with the anti-PDK-1 antibody coupled to protein A/G-agarose with or without co-incubation with 50 μg ml^{-1} of protamine sulfate. The immunoprecipitates were analyzed using SDS-PAGE and immunoblotting. Co-immunoprecipitaed full-length and the catalytic domain of protein kinase C βII were detected on the blot with the anti-PKC βII antibody, and PDK-1 in the immune complexes was detected using an anti-PDK-1 antibody.

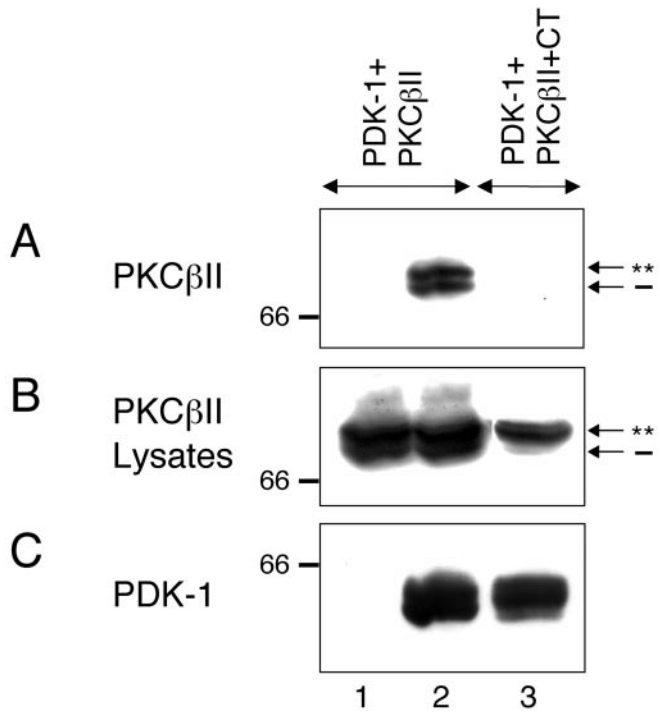

FIG. 8. **Co-expression of the COOH terminus of protein kinase C βII promotes the autophosphorylation of the full-length protein kinase C βII.** TsA201 cells were transiently transfected with Myc-tagged PDK-1 and protein kinase C βII in the absence (*lanes 1* and *2*) or presence (*lane 3*) of GST-CT. Detergent-solubilized cell lysates were immunoprecipitated with the anti-Myc antibody coupled to protein A/G-agarose (*lanes 2* and *3*) or the protein A/G-agarose alone (*lane 1*). *A,* the presence of protein kinase C βII in the immunoprecipitates was detected using the anti-PKC βII antibody. *B,* the amount of protein kinase C βII in 10% of the cell lysates was analyzed using immunoblotting with the anti-PKC βII antibody. *C,* the presence of PDK-1 in the immunoprecipitates was detected by the anti-Myc antibody.

kinase C upon expression of the COOH terminus promotes the autophosphorylation reactions which cause the mobility shift.

DISCUSSION

Co-immunoprecipitation studies reveal that PDK-1 associates preferentially with its substrate, unphosphorylated protein kinase C. Co-expression of specific domains of protein kinase C reveals that the primary determinants that regulate the interaction of protein kinase C with PDK-1 lie in the COOH terminus of protein kinase C, although weak contact with the C1 domain in the regulatory moiety is present. Importantly, the COOH terminus of protein kinase C provides a major determinant for binding PDK-1, and constructs of this domain displace PDK-1 from unphosphorylated protein kinase C, accelerating its maturation. These results show two points of regulation of protein kinase C by PDK-1: by directly phosphorylating the activation loop, and by indirectly controlling the autophosphorylation of the COOH terminus.

PDK-1 Preferentially Binds Unphosphorylated Protein Kinase C—Analysis of the interaction of PDK-1 with wild-type protein kinase C or phosphorylation site mutants reveals that PDK-1 preferentially binds unphosphorylated protein kinase C compared with phosphorylated, but inactive, enzyme. Studies with pure proteins reveal that the interaction is direct. These results suggest that either the conformation of unphosphorylated protein kinase C is required for optimal interaction with PDK-1, or that phosphate at specific positions sterically or electrostatically disrupts the interaction. The decreased binding of phosphorylated enzyme is unlikely to result from phosphate on the activation loop: the unphosphorylated species of T500E mutant (*i.e.* negative charge at the activation loop resulting from mutation but no phosphorylation of COOH-terminal sites) interacted to comparable levels with PDK-1 as unphosphorylated wild-type enzyme (data not shown). This led us to explore the role of phosphate at the carboxyl terminus, and of conformation, in regulating the interaction of protein kinase C with PDK-1.

Involvement of the Carboxyl Terminus in Regulating the Interaction of PDK-1 with Protein Kinase C—Studies with GST fusion constructs have revealed that the COOH terminus is a critical determinant in the interaction of protein kinase C with PDK-1. Our data reveal two seemingly contradictory findings: unphosphorylated PKC is much more effectively co-immunoprecipitated with PDK-1 compared with phosphorylated protein kinase C, yet constructs of the isolated carboxyl terminus are much more effective at disrupting the protein kinase C/PDK-1 interaction when phosphorylated. One explanation consistent with both of these findings is that phosphorylation of the carboxyl terminus of protein kinase C causes it to alter its conformation in such a way as to mask it. Consistent with this, inducing the activating open conformation of protein kinase C greatly increases the affinity of phosphorylated protein kinase C for PDK-1. The hypothesis that the COOH terminus is masked in the inactive conformation of mature protein kinase C is also supported by the finding that the phosphatase sensitivity of protein kinase C increases by 2 orders of magnitude upon adopting the active conformation (28). Thus, the interac-

tion of PDK-1 with protein kinase C is regulated in opposing ways by the phosphorylation state of the COOH terminus (with phosphate promoting tighter binding) and the conformation of the full-length protein (with the inactive conformation inhibiting binding). Consistent with this, the mature species of the S660A construct binds less tightly to PDK-1 than mature wild-type protein kinase, which has a phosphate at position 660.

Release of PDK-1 from Protein Kinase C Promotes Carboxyl-terminal Autophosphorylation—Carboxyl-terminal constructs bearing negative charge (PIF and phosphorylated carboxyl terminus of protein kinase C)[5] not only dramatically reduced the amount of protein kinase C present in the PDK-1 immune complexes, but they also increased the fraction of fully phosphorylated protein kinase C in cells. Thus, these constructs did not abolish the interaction of protein kinase C with PDK-1, because phosphorylation on the activation loop still occurred. Rather, by competing for binding to protein kinase C, these constructs promoted the maturation of protein kinase C. This result suggests that displacement of PDK-1 from protein kinase C accelerates the maturation of protein kinase C by promoting autophosphorylation.

A series of recent reports from Alessi and co-workers (23) have also implicated the hydrophobic phosphorylation motif as an important determinant in the regulation of PDK-1 activity. Using a yeast-two hybrid screen, this group identified a region in the COOH terminus of the protein kinase C-related kinase, PRK-2, that bound PDK-1 and named it PIF for PDK-1 interacting fragment (23). This region encompasses the hydrophobic phosphorylation motif except that Asp replaces the Ser at the potential phosphoacceptor position. Co-transfection studies with GST constructs of PIF revealed that Asp at the phosphoacceptor position and the flanking Phe were critical for efficient co-precipitation of PDK-1. The authors showed that inclusion of PIF in phosphorylation assays of Akt and PDK-1 promoted phosphate incorporation into the Ser^{473} of the hydrophobic phosphorylation motif of Akt. The interpretation of this result was that the interaction of PDK-1 with PIF causes an unprecedented change in substrate specificity causing PDK-1 to gain "PDK-2" activity and phosphorylate Ser^{473}.

Our data unveil an alternate explanation. The binding of PDK-1 to Akt could mask Ser^{473} which, like protein kinase C, is regulated by autophosphorylation (29). Addition of PIF would effectively compete with binding because PDK-1 has a much higher affinity for PIF, which has a negative charge at the corresponding phosphoacceptor position of the hydrophobic motif, than it does for the COOH terminus of Akt. Release of PDK-1 would then allow autophosphorylation. In support of this, Alessi and co-workers (30) reported that kinase-inactive constructs of Akt are phosphorylated at the activation loop but not Ser^{473} in the presence of the PDK-1·PIF complex. This is consistent with our data showing that phosphorylation of Ser^{473} requires the intrinsic catalytic activity of Akt because this site is modified by autophosphorylation. In addition, the basal phosphorylation of Ser^{473} is increased in ES cells from PDK-1 knock-outs, as would be expected if PDK-1 is no longer blocking the access of the carboxyl terminus to the active site (31). (Note that, like most kinases, Akt has some basal activity even without activation loop phosphorylation; constructs of Akt with Ala instead of Thr at the activation loop have residual activity (32).) Thus, by analogy with protein kinase C, the PDK-2-like activity observed in the presence of PIF likely arises from the ability of PIF to displace PDK-1 from Akt and expose the hydrophobic motif of Akt to autophosphorylation.

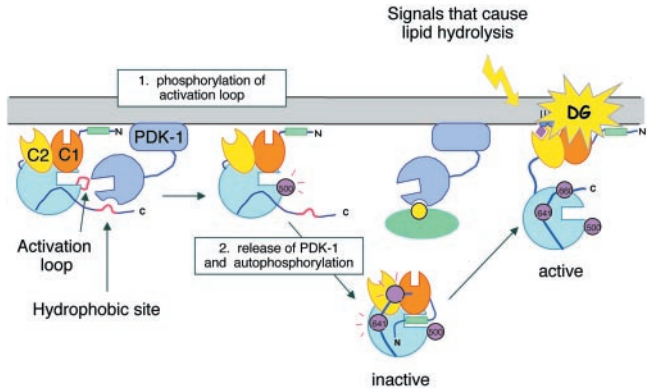

FIG. 9. **Model describing the regulation of protein kinase C βII by PDK-1.** PDK-1 binds newly synthesized, unphosphorylated protein kinase C primarily by interaction with the C terminus of protein kinase C (*left panel*). In this conformation, the pseudosubstrate of protein kinase C (*green rectangle*) is exposed, allowing access of PDK-1 to the activation loop phosphorylation site (26). PDK-1 then phosphorylates Thr^{500} of the activation loop and is released from protein kinase C (*middle panel*). The COOH terminus is then free to access the active site and becomes phosphorylated by an intramolecular reaction (20). *Pink circles* represent phosphate. Release of PDK-1 can be accelerated by phosphorylated versions of the carboxyl terminus (*green oval* with *yellow circle*) (*e.g.* 'PIF'-like molecules (23)) since PDK-1 has a higher affinity for the isolated carboxyl-terminal constructs that have negative charge at the phosphoacceptor position in the hydrophobic phosphorylation motif. The autophosphorylated COOH terminus then repositions itself in a manner which locks protein kinase C in a stable, catalytically competent conformation (*middle panel, bottom*). In this conformation, the COOH terminus becomes less accessible to interaction with PDK-1. Following activation of protein kinase C (*right panel*), the pseudosubstrate is released from the active site and accompanying conformational changes once again allow efficient binding of PDK-1 (not shown for simplicity).

More evidence for the importance of the carboxyl terminus in anchoring PDK-1 to its substrates derives from a second yeast two-hybrid screen which identified the COOH terminus of PKA as a sequence recognized by PDK-1 (30). The COOH terminus of PKA contains the turn motif phosphorylated in protein kinase C and Akt, but ends at the Phe directly preceding the Ser at the phosphoacceptor position of the hydrophobic phosphorylation motif (29). This terminal Phe helps anchor the phosphorylated COOH terminus on the upper lobe of the kinase core by binding a hydrophobic pocket on the back of the upper lobe (33). Residues contained in this pocket are conserved in PDK-1, leading Alessi and co-workers (30) to suggest that PDK-1, which does not have a hydrophobic phosphorylation motif, has an unoccupied hydrophobic pocket which binds PIF and other hydrophobic motifs. Related to this finding, a recent study has shown that the COOH terminus of protein kinase Cζ, which, like PRK-2, contains an acidic residue at its hydrophobic phosphorylation motif, is a docking site for PDK-1 (30). Similarly, phosphorylation of the hydrophobic site on the 90-kDa ribosomal S6 kinase-2 has recently been shown to provide a docking site for PDK-1 (34). The above reports converge on a key role for the hydrophobic motif in regulating the interaction of PDK-1 with its substrates.

Model—Taken together with previous findings, the data in this contribution lead us to propose the following model for the interaction of PDK-1 with protein kinase C. PDK-1 associates with newly synthesized, unphosphorylated protein kinase C. This interaction is mediated primarily through the COOH terminus of protein kinase C, with the hydrophobic phosphorylation motif playing a key role in the tethering of PDK-1. PDK-1

[5] Although PIF contains an acidic residue (Asp) at the phosphoacceptor position in the hydrophobic motif, COOH-terminal constructs of protein kinase C with Ser^{660} mutated to Glu were much less effective than PIF in binding PDK-1. Effective binding required phosphate at position 660. Note that PIF contains an additional acidic residue at the P-2 position, so the double negative charge of phosphate may be required to mimic the PIF sequence.

then phosphorylates protein kinase C βII on Thr^{500} of the activation loop, the first and rate-limiting step in the processing of protein kinase C by phosphorylation (9) (Fig. 9, *left panel*). PDK-1 then dissociates from protein kinase C (Fig. 9, *middle panel*), unmasking the carboxyl terminus and making it available for the intramolecular autophosphorylation of Thr^{641} and Ser^{660} (20). The phosphorylated COOH terminus shifts its position in such a way as to become masked to PDK-1 (Fig. 9, *bottom panel*). Thus, although PDK-1 has intrinsically a much higher affinity for the isolated COOH terminus when it is phosphorylated compared with unphosphorylated, its apparent affinity for the phosphorylated COOH terminus is reduced in the context of the mature, inactive, protein kinase C. Activation of the mature enzyme results in conformational changes that once again expose the COOH terminus for more efficient binding to PDK-1. However, more studies are required to elucidate the role of protein kinase C activation in binding to PDK-1 in a cellular context.

The release of PDK-1 from protein kinase C is greatly accelerated by the presence of isolated COOH-terminal fragments that contain negative charge at the hydrophobic motif such as PIF or the phosphorylated carboxyl terminus of protein kinase C βII. Whether there are accessible phosphorylated or Glu-containing hydrophobic sequences *in vivo* that regulate the interaction of PDK-1 with protein kinase C remains to be explored. If so, this could provide a regulatory mechanism for fine-tuning phosphorylation at the COOH terminus of protein kinase C. One interesting possibility is that the activated conformation of protein kinase C serves as a sink for PDK-1, since this conformation has an exposed, phosphorylated COOH terminus that is highly effective at binding PDK-1. If this is the case, activation of protein kinase C would be predicted to modulate the processing of newly synthesized protein kinase C. Curiously, once phosphorylated at the COOH terminus, phosphorylation of the activation loop has little effect on either the basal or cofactor stimulated activity of the mature enzyme *in vitro* (9). This suggests that the interaction of activated protein kinase C with PDK-1 is not likely to directly regulate the catalytic function of protein kinase C. Further study is required to elucidate whether activation of protein kinase C *in vivo* does, indeed, promote PDK-1 binding and, if so, what the functional consequences of this interaction are.

In summary, our study has identified the COOH terminus as a key determinant in regulating the interaction of protein kinase C and PDK-1 *in vivo*. Our data suggest that the carboxyl terminus of unphosphorylated protein kinase C docks PDK-1, with release of PDK-1 unmasking the carboxyl terminus to allow autophosphorylation. This release is promoted by phosphorylated versions of the carboxyl terminus, which effectively compete for binding to PDK-1 given the higher intrinsic affinity of PDK-1 for negative charge at the hydrophobic motif. This model could account not only for why PIF promotes phosphorylation of the hydrophobic site of Akt, but also why in attempts to purify a putative upstream kinase for conventional protein kinase Cs, protein kinase Cζ (which contains an acidic residue at its hydrophobic site and so might be expected to displace PDK-1 from conventional protein kinase Cs) was identified in a protein complex (35).

The above results suggest two key steps in the regulation of protein kinase C by PDK-1: 1) phosphorylation of the activation loop which is regulated by the activity of PDK-1, and 2) autophosphorylation of the COOH-terminal sites, including the hydrophobic site, which is regulated by release of PDK-1 from protein kinase C. The tight coupling of COOH-terminal autophosphorylation to the activation loop phosphorylation catalyzed by PDK-1 likely accounts for the confusion in the literature regarding whether PDK-1 or PDK-1-like (PDK-2) kinases phosphorylates this site (4, 23, 36).

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