The Phosphoinositide-dependent Kinase, PDK-1, Phosphorylates Conventional Protein Kinase C Isozymes by a Mechanism That Is Independent of Phosphoinositide 3-Kinase*

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Phosphorylation by the phosphoinositide-dependent kinase, PDK-1, is required for the activation of diverse members of the AGC family of protein kinases, including the protein kinase C (PKC) isozymes. Here we explore the subcellular location of the PDK-1-mediated phosphorylation of conventional PKCs, and we address whether this phosphorylation is regulated by phosphoinositide 3-kinase. Pulse-chase experiments reveal that newly synthesized endogenous PKC α is primarily phosphorylated in the membrane fraction of COS-7 cells, where it is processed to a species that is phosphorylated at the activation loop and at two carboxyl-terminal positions. This "mature" species is then released into the cytosol. Deletion of the plekstrin homology domain of PDK-1 results in a 4-fold increase in the rate of processing of PKC indicating an autoinhibitory role for this domain. Autoinhibition by the plekstrin homology domain is not relieved by binding 3′-phosphoinositides; PKC is phosphorylated at a similar rate in serum-treated cells and serum-starved cells treated with the phosphoinositide 3-kinase inhibitors, LY294002 and wortmannin. Under the same conditions, the PDK-1-catalyzed phosphorylation of another substrate, Akt/protein kinase B, is abolished by these inhibitors. Our data are consistent with a model in which PDK-1 phosphorylates newly synthesized PKC by a mechanism that is independent of 3′-phosphoinositides.

Phosphorylation at a segment near the active site, the activation loop, critically regulates the function of diverse members of the protein kinase superfamily. Mounting evidence suggests that for AGC kinase family members, a single kinase, the phosphoinositide-dependent kinase, PDK-1,[1] assumes this role (1, 2). Originally discovered as the upstream kinase for Akt/protein kinase B (3), PDK-1 is now known to phosphorylate many other kinases, including PKC, p70S6 kinase, ribosomal S6 kinase, p21-activated kinase, PKC-related kinase, and serum glucocorticoid kinase (1, 2, 4). The ability to regulate the function of multiple protein kinases places PDK-1 at a pivotal point in cellular signaling. Thus, understanding how the cellular function of PDK-1 is regulated is a central question in signal transduction.

Signaling by PKC has been intensely studied in the past 2 decades, spawned by the seminal discovery of Nishizuka and co-workers (5) that this member of the kinase superfamily transduces the myriad of signals that promote phospholipid hydrolysis. Yet the fact that all family members depend on a series of ordered phosphorylation events before they are competent to respond to lipid second messengers has only recently been appreciated (6, 7). The discovery that PKC isozymes are processed by phosphorylation at three conserved positions (8, 9) led to a search for an upstream kinase. This search culminated in the finding that PDK-1, discovered in 1997 as the upstream kinase for the close cousin of PKC, Akt/protein kinase B, is the activation loop kinase for conventional (10), novel, and atypical PKCs (11, 12). Thus, PKC is regulated by two mechanisms, phosphorylation and cofactors.

Protein kinase C isozymes consist of a single polypeptide with the carboxyl-terminal moiety containing the kinase core and the amino-terminal moiety containing one (C1) or two (C1 and C2) membrane-targeting modules coupled to an autoinhibitory pseudosubstrate sequence. The dogma for regulation of PKC is that generation of diacylglycerol engages the C1 domain on membranes, an interaction that is facilitated by Ca^{2+}-triggered binding of the C2 domain for some isozymes (13). This membrane interaction provides the energy to release the autoinhibitory pseudosubstrate sequence from the substrate-binding cavity, resulting in substrate phosphorylation and downstream signaling. There are three classes of PKC isozymes: conventional isozymes (α, βI, βII, and γ) have a C1 and C2 domain and are regulated by diacylglycerol and Ca^{2+}; novel isozymes (δ, ϵ, and η/L) have a C1 domain and a "novel" C2 domain and are regulated by diacylglycerol but not Ca^{2+}; and atypical isozymes (ζ, ι/λ) contain an "atypical" C1 domain that is not competent to bind diacylglycerol so that the function of atypical PKCs is not regulated by the classical ligands Ca^{2+} and diacylglycerol. The kinase domain contains three conserved phosphorylation sites: the activation loop (Thr-500 in PKC βII) which is phosphorylated by PDK-1, and two carboxyl-terminal sites, the turn and hydrophobic motif (Thr-641 and Ser-660, respectively, in PKC βII), which are regulated by autophosphorylation in conventional PKCs (14). These sites are conserved in all isozymes except for atypical PKCs, which contain a phospho-mimic, Glu, at the phospho-acceptor position of the hydrophobic motif. Phosphate at the activation loop unmasks the substrate-binding cavity and structures the enzyme for catalysis, with phosphates at the two carboxyl-termi-

[1] The abbreviations used are: PDK-1, phosphoinositide-dependent kinase 1; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; PH, plekstrin homology; HA, hemagglutinin; GFP, green fluorescent protein.

nal positions then locking the enzyme in this catalytically competent conformation. The fully phosphorylated species is the mature form that is competent to respond to lipid second messengers.

The regulation of PKC by PDK-1 begs the question as to whether PKC isozymes are under the coordinated regulation of two lipid signaling pathways: the PI3-kinase pathway that would control the maturation of PKC, and the phospholipase pathway that allosterically controls the activity of the mature enzyme. PDK-1 contains a PH domain, and binding studies suggest it has a high intrinsic affinity for 3′-phosphoinositides (3, 15, 16). Nonetheless, *in vitro* analyses reveal that PDK-1 has a high basal activity in the absence of any cofactors, with the phosphorylation of substrates such as PKC being unaffected by the presence of phosphoinositides (10). One possibility is that PDK-1 is constitutively active in cells, with specificity in signaling by PDK-1 dictated by the conformation of its substrates (1). Thus, particular signals would target downstream kinases for phosphorylation by PDK-1 by altering their conformation or subcellular location for recognition by PDK-1. Indeed, PDK-1 holds its name because of the dependence on phosphoinositides for its first-discovered substrate, Akt/protein kinase B, to unmask the PDK-1 phosphorylation site (3). Specifically, generation of 3′-phosphoinositides recruits Akt to the membrane by its PH domain, an event that unmasks the activation loop. Deletion of the PH domain or forced recruitment of Akt to membranes by addition of other membrane-targeting modules results in constitutive phosphorylation of Akt (17–19). The conformation of PKC is also critical for phosphorylation by PDK-1; the pseudosubstrate must be removed from the active site in order for PDK-1 to access the activation loop (20). However, it is unclear whether the intrinsic activity of PDK-1 is regulated *in vivo*.

This study examines the subcellular location, mechanism, and regulation of the phosphorylation of conventional PKCs by PDK-1. Pulse-chase experiments reveal that newly synthesized endogenous PKC α is phosphorylated by PDK-1 in a membrane fraction. Studies with wild-type and phosphorylation site mutants of PKC α or βII reveal that this phosphorylation is the first step in the processing of PKC, is under negative regulation by the PH domain of PDK-1, and is independent of PI3-kinase activity.

EXPERIMENTAL PROCEDURES

Materials—Easy Tag [^{35}S]methionine/cysteine (1175 Ci mmol^{-1}) was purchased from PerkinElmer Life Sciences. Methionine/cysteine-deficient DMEM and standard DMEM were purchased from BioWhittaker. Fetal bovine serum and calf serum were from Omega Scientific Inc. Polyvinylidene difluoride membranes were from Millipore. Polyclonal antibodies against PKC α or βII, and protein A/G-agarose were obtained from Santa Cruz Biotechnology. A monoclonal antibody against PKC α was purchased from Transduction Laboratories; a polyclonal phospho-specific antibody (P500) that recognizes the phosphorylated activation loop of PKC isozymes was generated and characterized as described previously (10, 21). Phospho-specific antibodies, P660 and P308, which recognize the phosphorylated hydrophobic motif of PKC isozymes (Ser-660 in PKC βII) and the phosphorylated activation loop of Akt (Thr-308) respectively, were purchased from Cell Signaling. Wortmannin and LY294002 were purchased from Calbiochem. All other chemicals were reagent-grade.

Cell Fractionation—For experiments involving separation of the cytosol and membrane fractions, COS-7 cells were resuspended in lysis buffer (20 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 100 nM microcystin) and subjected to sonication (Branson bath sonicator). Whole cell lysates were centrifuged at $100,000 \times g$ for 20 min at 4 °C, and the resulting supernatant (cytosol) was removed. The pellet was resuspended in 500 μl of lysis buffer containing 1% Triton X-100 and centrifuged at $100,000 \times g$ for 20 min at 4 °C; the resulting supernatant comprised the detergent-soluble membrane fraction. The Triton X-100-insoluble pellet was resuspended

in lysis buffer containing 0.5% SDS, briefly probe-sonicated, and the SDS-soluble fraction separated by centrifugation at $100,000 \times g$ for 20 min at 4 °C. For some experiments, cells were lysed in lysis buffer containing 1% Triton X-100 and centrifuged at $100,000 \times g$ for 20 min at 4 °C to yield a Triton X-100-soluble supernatant containing cytosolic and detergent-soluble membrane proteins.

Pulse-Chase Labeling—COS-7 cells were plated to 50% confluency in 6-well dishes 1 day before transfection. Unless otherwise indicated, cells were maintained in DMEM containing 10% fetal bovine serum. Cells were transfected with one of the following expression constructs: bovine PKC α (gift of Yusuf Hannun, Medical University of South Carolina), rat PKC βII, mutant constructs of rat PKC βII (T497A/T498A/T500A (referred to as T500AAA), T641E, S660A, or S660E (20, 22, 23)), amino-terminal Myc-tagged rat PKC ζ (gift of Alex Toker, Harvard Medical School), or murine Akt α with an amino-terminal HA tag (gift of Alex Toker, Harvard Medical School) using the Superfect Transfection reagent (Qiagen). Untransfected COS-7 cells were used to study the endogenous PKC α. Approximately 24–30 h post-transfection, cells were incubated with methionine/cysteine-deficient DMEM for 15 min at 37 °C. The cells were then pulse-labeled with 0.5 mCi of [^{35}S]methionine/cysteine in methionine/cysteine-deficient DMEM for 7 min at 37 °C and chased in chase media (DMEM containing 5 mM unlabeled methionine and 5 mM unlabeled cysteine) for 0–60 min as indicated in the specific experiments. At the times indicated in the figure legends, cells were harvested, lysed, and fractionated as described above. For experiments in which PI3-kinase was inhibited, NIH 3T3 cells were used; these were transfected using Effectene Transfection reagents (Qiagen). Following transfection, one-half of the transfected cells was incubated in serum-free DMEM overnight, whereas the other half of the cells was kept in serum-containing growth media (DMEM + 10% calf serum). Approximately 30 h post-transfection, serum-starved cells were treated with wortmannin (100 nM) and LY290004 (30 μM) for 20 min at 37 °C. The cells were then labeled with [^{35}S]methionine/cysteine and chased for 0–90 min as indicated in the specific experiments.

Immunoprecipitation—All constructs of PKC α and βII were immunoprecipitated with an anti-PKC α monoclonal antibody that recognizes both α and βII isozymes (Transduction Laboratories). An anti-Myc antibody and an anti-HA antibody were used to immunoprecipitate the Myc-tagged PKC ζ and the HA-tagged Akt, respectively. COS-7 or NIH 3T3 cell lysates were immunoprecipitated in the presence of 100 μl of protein A/G-agarose and 1 μl of specific antibodies for 16 h at 4 °C. Agarose beads were washed 3 times with 300 μl of lysis buffer containing 1% Triton X-100 and twice with 300 μl of 0.5 M NaCl. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon. The ^{35}S signals were obtained by exposing Immobilon membranes to a Bio-Rad molecular imaging screen for 72 h, and PKC (or Akt) was detected by Western blot analysis using chemiluminescence. Relative radioactivity and chemiluminescence were quantified by analysis using a Molecular Imager System (Bio-Rad) or by a CCD camera using a GeneGnome bioimaging system (Syngene), respectively.

RESULTS

Endogenous Protein Kinase C α Is Phosphorylated in a Detergent-soluble Membrane Fraction following Its Biosynthesis—To determine where in the cell newly synthesized PKC is phosphorylated, we employed a pulse-chase analysis that took advantage of the mobility shift accompanying phosphorylation of PKC (6, 8). Specifically, we asked the following question: in which cellular fraction does the faster migrating, unphosphorylated species of PKC partition? To ensure that we were examining the physiologically relevant location, initial studies probed the PKC α endogenous to COS-7 cells. Cells were incubated with [^{35}S]methionine/cysteine for 7 min to label a discrete population of newly synthesized protein. This population was then chased by incubation of cells with unlabeled methionine/cysteine for various times. Cells were lysed in the absence of detergent and fractionated into a cytosolic and particulate fraction. The particulate fraction was then extracted with Triton X-100 to yield a detergent-soluble membrane fraction, and the phosphorylation of PKC in each fraction was monitored by examining the electrophoretic mobility of immunoprecipitated kinase on SDS-PAGE.

The autoradiogram in Fig. 1 (*top panel*) shows that ^{35}S-labeled PKC was present in both the membrane and cytosolic
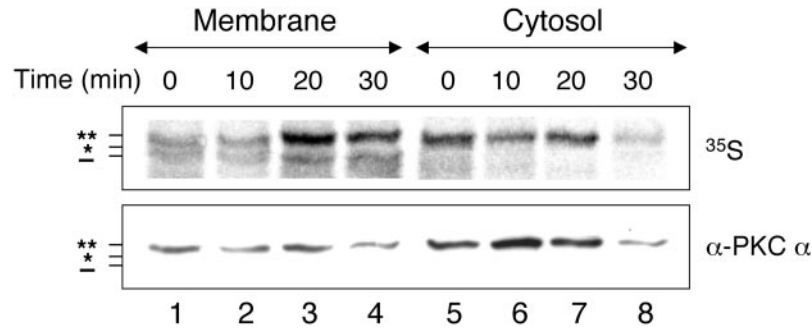
FIG. 1. **Endogenous PKC α associates with the Triton-soluble membrane fraction of COS-7 cells following its biosynthesis.** COS-7 cells were metabolically labeled with [^{35}S]methionine/cysteine for 7 min and chased for 0 (*lanes 1* and *5*), 10 (*lanes 2* and *6*), 20 (*lanes 3* and *7*), or 30 min (*lanes 4* and *8*) with unlabeled methionine/cysteine. Cells were lysed and fractionated into membrane (*lanes 1–4*) and cytosolic (*lanes 5–8*) fractions and immunoprecipitated with an anti-PKC α monoclonal antibody. The *upper panel* shows the autoradiogram of the immunoprecipitated ^{35}S-labeled PKC α; the *lower panel* shows the corresponding Western blot probed with an anti-PKC α polyclonal antibody. Unphosphorylated PKC is indicated with a *dash*; the intermediate species (phosphate on the activation loop and one of the two carboxyl-terminal phosphorylation sites) is indicated with *one asterisk*, and the mature species (phosphates on all three processing sites) is labeled with *two asterisks*.

fractions. No PKC was present in the detergent-insoluble fraction (not shown). Immediately following the pulse of [^{35}S]methionine/cysteine, PKC migrated as two major bands in the membrane fraction (*lane 1*). Previous mass spectrometric analysis has revealed that the faster migrating band (*dash*) corresponds to unphosphorylated protein; a barely detectable intermediate band (*asterisk*) corresponds to protein phosphorylated at the activation loop and carboxyl-terminal turn motif, and the slowest migrating band (*double asterisk*) corresponds to protein phosphorylated at all three "maturation" positions: the activation loop, turn motif, and hydrophobic motif (8). There were comparable amounts of upper and lower bands immediately following the 7-min chase, indicating that the fastest migrating band was chased to the slower migrating band with a half-time on this order. This shift resulted from phosphorylation; treatment of sample enriched in the slower migrating species with protein phosphatase 1, which dephosphorylates all three *in vivo* phosphorylation sites (8), caused the enzyme to migrate as a single, faster migrating species (data not shown). Newly synthesized unphosphorylated PKC localized exclusively to the membrane fraction; even at the earliest chase time (*lane 5*), there was no detectable faster-migrating band in the cytosolic fraction. Rather, phosphorylated PKC accumulated in the cytosol. Western blot analysis to probe for the total pool of PKC revealed that ~70% of the PKC was present in the cytosolic fraction and ~30% in the membrane fraction under the conditions of these experiments (Fig. 1, *lower panel*). Note that only the mature form (indicated with *double asterisk*) was detected by Western blot analysis, revealing that >95% of the bulk PKC α in COS-7 cells is fully processed under standard cell culture conditions (*i.e.* with serum, so that PI3-K is activated, but in the absence of phospholipase C activators). These data reveal that the physiologically relevant location for processing of endogenous PKC α is at a membrane fraction. Following phosphorylation, the majority of the PKC is released to the cytosol.

Overexpressed Protein Kinase C α Localizes to the Membrane and Cytosol following Its Biosynthesis—Overexpression of PKC often results in localization of unphosphorylated species in the Triton X-100-insoluble fraction (*e.g.* Ref. 22). Thus, we asked whether overexpressed PKC α was processed in the same way as endogenous PKC α or whether newly synthesized enzyme accumulated in the detergent-insoluble fraction. COS-7 cells transfected with the cDNA for PKC α were subjected to pulse-chase analysis, lysed in the presence of Triton X-100, and separated into Triton X-100-soluble (*i.e.* cytosol plus membrane) and -insoluble (*i.e.* cytoskeletal) fractions. The insoluble fraction was further solubilized with SDS to yield an SDS-soluble fraction. Under the conditions of our experiments, PKC
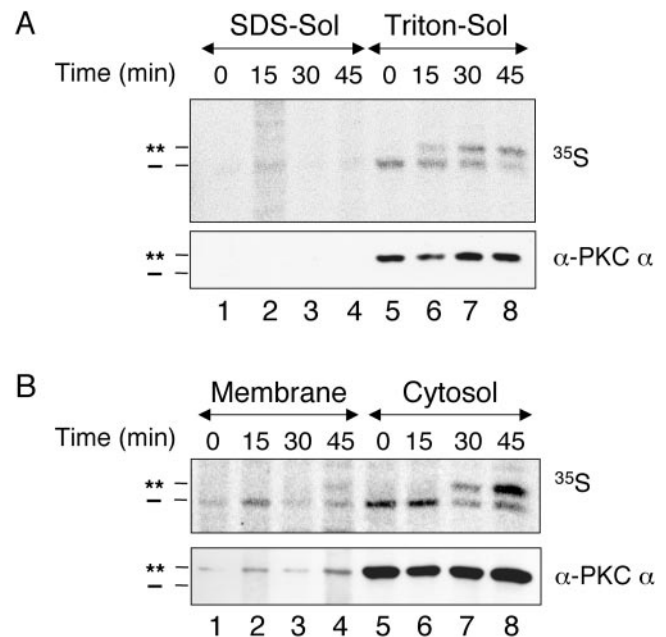

FIG. 2. **Overexpressed protein C α associates with both the membrane and cytosolic fractions following its biosynthesis.** COS-7 cells were transfected with PKC α and pulse-labeled with [^{35}S]methionine/cysteine. The cells were then chased for 0 (*lanes 1* and *5*), 15 (*lanes 2* and *6*), 30 (*lanes 3* and *7*), and 45 min (*lanes 4* and *8*). *A,* cells were fractionated into Triton X-100-soluble (*Triton-Sol*) and Triton X-100-insoluble, but SDS-soluble (*SDS-Sol*), fractions. The *upper panel* shows the autoradiogram of the immunoprecipitated ^{35}S-labeled PKC α, and the *lower panel* shows the corresponding Western blot. *B,* cells were fractionated into cytosol and Triton X-100-soluble membrane fractions. The *upper panel* shows the autoradiograph of the immunoprecipitated ^{35}S-labeled PKC α, and the *lower panel* shows the corresponding Western blot. Unphosphorylated PKC is labeled with a *dash*, and the mature species (phosphates on all three processing sites) is labeled with *two asterisks*.

was overexpressed ~10-fold relative to the endogenous PKC. The autoradiogram in Fig. 2*A* shows that newly synthesized PKC localized to the Triton X-100-soluble fraction, with no detectable ^{35}S-labeled protein in the SDS-soluble fraction. This species migrated as a 78-kDa band at the first time point (immediately following the chase; *lane 5*) and shifted to a slower migrating species with a half-time between 15 (*lane 6*) and 30 (*lane 7*) min. The ^{35}S-labeled pool of PKC was almost completely phosphorylated following 45 min of chase (*lane 8*). Western blot analysis revealed that the Triton X-100-soluble fraction contained only the slower mobility, phosphorylated species of PKC, consistent with previous results (8). In sum-

mary, these pulse-chase data show that the SDS-soluble fraction is devoid of any newly synthesized PKC; rather, recombinant PKC α is processed to a fully phosphorylated species in a Triton X-100-soluble fraction. Studies with PKC βII revealed similar results; recombinant enzyme was processed exclusively in the detergent-soluble fraction (note that this isozyme was processed with a slightly longer half-time of around 60 min (*e.g.* Fig. 5)).

Analysis of the newly synthesized recombinant PKC α in the membrane and cytosolic fractions revealed that the processing of exogenous kinase differed from that of endogenous kinase in two ways. First, newly synthesized unphosphorylated PKC was present in both the membrane (Fig. 2*B, lanes 1–4*) and cytosolic (Fig. 2*B, lanes 5–8*) fractions (in contrast to just the membrane fraction for exogenous protein). Second, newly synthesized phosphorylated protein was not detected in the membrane fraction but partitioned exclusively in the cytosolic fraction (in contrast to both fractions for endogenous protein). One possibility for these differences is that potential docking sites at the membrane for newly synthesized PKC are saturated in the overexpression studies, so that excess newly synthesized enzyme accumulates in the cytosol. Once the membrane-associated population is phosphorylated and released into the cytosol, the excess enzyme could bind the membrane and be processed. Alternatively, this mislocalized enzyme could be phosphorylated in the cytosol; although we were unable to detect endogenous PDK-1 with our antibodies, the bulk of recombinant PDK-1 localizes to the cytosol under our cell culture conditions. Western blot analysis revealed that the majority (85%) of the mature, phosphorylated species of PKC was present in the cytosolic fraction (Fig. 2*B, lower panel*) suggesting that once phosphorylated, PKC accumulates in the cytosol.

The PH Domain of PDK-1 Is Not Required for the Processing of Protein Kinase C—We next explored whether the membrane localization of PDK-1 was necessary for efficient phosphorylation of PKC. To this end, we examined the rate of processing of PKC by a construct of PDK-1 deleted in its membrane-targeting module, the PH domain. Pulse-chase experiments were performed on COS-7 cells transfected with PKC βII and either wild-type PDK-1 or a construct in which the PH domain had been deleted. Confocal microscopy confirmed that the deletion mutant did not associate with the plasma membrane of stimulated NIH 3T3 cells (data not shown). Co-expression of the wild-type PDK-1 (Fig. 3, *middle panel*) resulted in a modest acceleration of PKC maturation relative to the rate of maturation in cells containing only endogenous PDK-1 (*upper panel*). This suggests that endogenous PDK-1 is unlikely to be limiting for the processing of PKC and, indeed, that higher levels of PDK-1 are inhibitory. Fig. 3, *lower panel*, shows that deletion of the PH domain resulted in an increase in the rate of processing of PKC. Specifically, in cells co-transfected with wild-type PDK-1, ~40% of the PKC migrated as the fully phosphorylated form following 60 min of chase. In contrast, in cells co-transfected with the construct of PDK-1 lacking the PH domain, ~40% of the PKC migrated as the fully phosphorylated form following 15 min of chase. Thus, the PH domain is not required for the phosphorylation of PKC. In fact, the PH domain has an autoinhibitory function; removal of the domain increases the rate of processing of PKC. It remains to be determined whether this increased rate of processing occurs at the membrane or cytosol.

Phosphorylation by PDK-1 Is the First Step in the Maturation of Protein Kinase C—Mutation of the activation loop phosphorylation sites to non-phosphorylatable, neutral residues results in expression of PKC that is not phosphorylated and cannot be activated (20, 24, 25). To determine whether phosphorylation
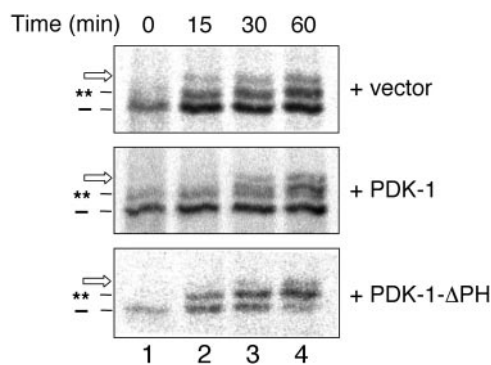

FIG. 3. **The PH domain of PDK-1 is not required for the processing of conventional PKC.** Autoradiograms showing the maturation of PKC from a pulse-chase analysis of COS-7 cells transfected with PKC βII and either vector (*top panel*) wild-type PDK-1 (*middle panel, + PDK-1*) or a construct of PDK-1 lacking the PH domain (*bottom panel; + PDK-1-ΔPH*). Cells were pulse-labeled for 7 min and chased for 0, 15, 30, or 60 min (*lanes 1–4,* respectively). Cells were lysed in buffer containing 1% Triton X-100 and centrifuged, and PKC βII was immunoprecipitated from the Triton X-100-soluble supernatants (cytosol and membrane). Unphosphorylated PKC is indicated with a *dash*, and the mature species (phosphates on all three processing sites) is labeled with *two asterisks*. The *open arrow* indicates the endogenous PKC α in the immunoprecipitates, which, upon full phosphorylation, migrates slightly slower than the fully phosphorylated βII isozyme.

by PDK-1 is a required first step in the maturation of PKC, or whether such constructs are processed by phosphorylation but then become rapidly dephosphorylated, we examined the processing of activation loop mutants by pulse-chase analysis. COS-7 cells were transfected with a mutant in which the activation loop threonine as well as two flanking threonines (497 and 498) had been mutated to Ala (referred to as T500AAA)[2] and subjected to pulse-chase analysis. Fig. 4 (*upper panel*) shows that replacement of the activation loop threonines with non-phosphorylatable residues prevented the characteristic electrophoretic mobility shift that PKC undergoes as a result of carboxyl-terminal phosphorylation (*lanes 1–4, upper panel*). Therefore, activation loop phosphorylation is the first step and required step in the maturation of PKC. These data reveal that phosphorylation at the activation loop is required for the subsequent autophosphorylation of the carboxyl-terminal sites.

We next addressed whether the phosphorylation by PDK-1 of the activation loop or of the carboxyl-terminal sites is the rate-limiting step in the maturation of PKC. To this end, we examined the rate of processing of phosphorylation site constructs of PKC βII in which carboxyl-terminal phosphorylation site residues were replaced with Glu. We have previously shown that Glu at the turn motif site, Thr-641, or the hydrophobic motif site, Ser-600, provides a reasonably effective mimic of phosphate (22, 23). Fig. 4 (*lower panel*) shows that the T641E mutant was phosphorylated with a half-time of ~30 min. This rate was double that of wild-type PKC βII (see Fig. 5; half-time of 60 min for wild-type PKC βII). Thus, the presence of negative charge at the turn motif caused a moderate enhancement in the rate of processing of PKC. This suggests that autophosphorylation at this position limits the rate of processing of PKC.

In contrast to the turn motif, mutation of the hydrophobic site to Glu did not alter the rate of processing of PKC βII. Fig. 5 shows that the S660E and wild-type enzyme were phosphorylated with similar kinetics: ~45% of the protein was chased

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[2] Note that pulse-chase analysis of the PKC mutant T500V resulted in a small fraction of PKC migrating at the slower mobility, indicating some compensatory phosphorylation of residues neighboring Thr-500 (data not shown), as has been reported for PKC α (25); this compensating phosphorylation was prevented in the triple mutant, T500AAA.
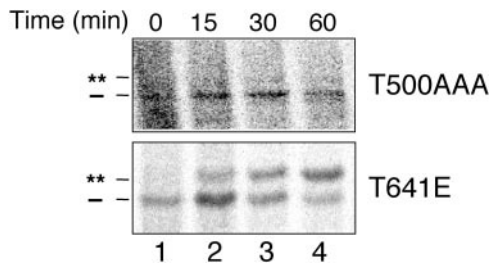
FIG. 4. **Pulse-chase analysis of phosphorylation site mutants of PKC βII.** Autoradiogram of ^{35}S-labeled PKC βII immunoprecipitated from COS-7 cells transfected with either T500AAA (*upper panel*) or T641E (*lower panel*) constructs and subjected to pulse-chase analysis. Cells were chased for 0, 15, 30, or 60 min (*lanes 1–4,* respectively), and the mutant PKCs were immunoprecipitated from the Triton X-100-soluble fraction. Unphosphorylated PKC is labeled with a *dash*, and the mature species (phosphates on all three processing sites) is labeled with *two asterisks.*

FIG. 5. **Phosphorylation of the activation loop is the rate-limiting step in the maturation of conventional PKC.** *A,* autoradiograms of ^{35}S-labeled PKC βII from COS-7 cells transfected with wild-type PKC βII (*top panel, WT*), S660E mutant (*middle panel*), and S660A mutant (*bottom panel*). Cells were pulse labeled for 7 min and chased for 0, 15, 30, and 60 min (*lanes 1–4,* respectively); PKC was immunoprecipitated from the Triton X-100-soluble fraction. Unphosphorylated PKC is labeled with a *dash*, and the mature species (phosphates on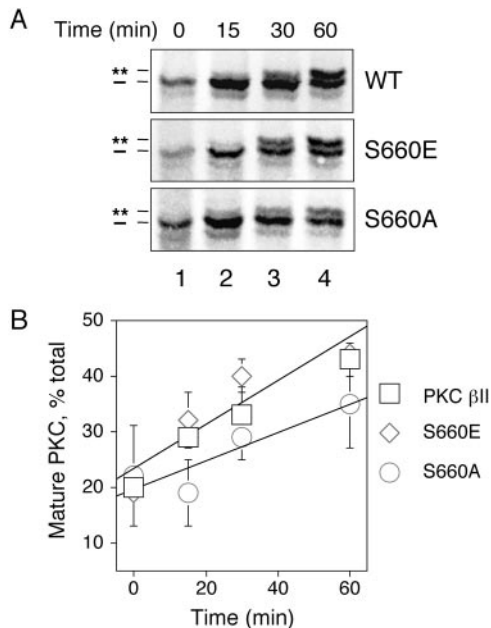 all three processing sites) is labeled with *two asterisks. B,* graph showing the percent of mature PKC as a function of chase time, obtained by quantifying the upper and lower bands of WT (*squares*), S660E (*diamonds*), and S660A (*circles*) PKC in *A.* Data represent the average and range of two experiments. Lines shown are a least squares fit for the data obtained for the two mutants.

to the slower migrating form following 60 min of chase. Mutation of Ser-660 to Ala decreased the rate of processing slightly, with only about 35% of the total PKC present as the slower-migrating species following 45 min of chase. Importantly, the S660E mutant was not processed at a faster rate than wild-type enzyme, revealing that phosphorylation of this position is not the rate-limiting step in the maturation of PKC. Western blot analysis revealed that the S660E and S660A protein in the Triton X-100-soluble fraction was fully phosphorylated and migrated as the slower migrating species (data not shown), as previously described (23). In summary, these data suggest that autophosphorylation of Thr-641 dictates the rate of processing of PKC, whereas the phosphorylation of Ser-660 immediately follows the autophosphorylation of Thr-641.

Processing of Protein Kinase C Is Not Regulated by PI3-Kinase—The finding that the PH domain of PDK-1 is not required for the phosphorylation of PKC led us to explore whether the phosphorylation of PKC is regulated by PI3-kinase. NIH 3T3 cells expressing PKC were treated with either serum to activate PI3-kinase or with the PI3-kinase inhibitors wortmannin and LY294002. Early passage NIH 3T3 cells were chosen for this analysis because PI3-kinase activity is effectively abolished in these cells with appropriate pharmacological intervention (11). The rate of phosphorylation of PKC βII was examined by pulse-chase analysis. In addition, the phosphorylation state of the bulk population of PKC was determined by Western blot analysis using a phospho-activation loop antibody, P500. These studies used GFP-tagged PKC βII in an effort to increase the signal in these cells (which have lower efficiency of expression compared with COS-7 cells) by increasing the specific activity of the ^{35}S labeling.

Fig. 6 shows the result of a pulse-chase experiment of NIH 3T3 cells expressing PKC βII. Immediately following the pulse, ^{35}S-labeled PKC migrated as a single band in serum-treated cells (*upper panel, lane 1*) and serum-starved cells treated with PI3-kinase inhibitors (*upper panel, lane 4*). Following 90 min of chase, ^{35}S-labeled PKC in cells from both treatments migrated as a doublet with bands of similar intensity (*upper panel, lanes 3* and *6*). This reveals that PKC was processed with a similar rate (half-time of ~90 min) in serum-activated cells and serum-starved cells treated with wortmannin and LY294002. Note that this half-time of processing is slower than that observed in COS-7 cells and likely reflects lower levels of PDK-1 in the NIH 3T3 cells. Consistent with the finding that inhibition of PI3-kinase had no detectable effect on the processing of PKC by phosphorylation, Western blot analysis of the immunoprecipitated PKC revealed robust activation loop phosphorylation in protein from cells treated with serum or with the PI3-kinase inhibitors (*middle panel*). In fact, quantitation of the degree of activation loop phosphorylation revealed that the PKC βII in cells treated with PI3-kinase inhibitors was elevated relative to the PKC in serum-treated cells (Fig. 6*B*). Note that the lower amount of total PKC in the wortmannin/LY294002-treated cells (*lower panel*) reflected the slowed growth rate and hence lower cell number resulting from growth of cells in the absence of serum.

The above results indicate that the processing of a conventional PKC is not regulated by PI3-kinase. To determine whether this constitutive processing is unique to conventional PKCs or a property shared by other isozymes, we examined the processing of an atypical PKC, PKC ζ. Analysis of the autoradiogram detecting newly synthesized PKC ζ showed that the processing of this isozyme was not accompanied by the defined decrease in electrophoretic mobility that accompanies the processing of PKC α and βII (Fig. 6*A, upper panel*). This could reflect the presence of a Glu at the hydrophobic phosphorylation motif, so that only one position at the carboxyl terminus contributes to the electrophoretic mobility shift. Nonetheless, Western blot analysis of the total pool of PKC in the immunoprecipitate using the P500 antibody revealed activation loop phosphorylation for PKC ζ from serum-treated as well as inhibitor-treated cells (Fig. 6*A, middle panel*). Quantitation of the amount of activation loop phosphorylation relative to total PKC ζ revealed that inhibition of PI3-kinase reduced activation loop phosphorylation ~2-fold (Fig. 6*B*). Thus, inhibition of PI3-kinase reduced, but did not abolish, the activation loop phosphorylation of PKC ζ.

Similar to overexpressed PKC βII, the maturation of endogenous PKC α was unaffected by serum starvation. Fig. 7 shows endogenous PKC α from serum-starved NIH 3T3 cells was
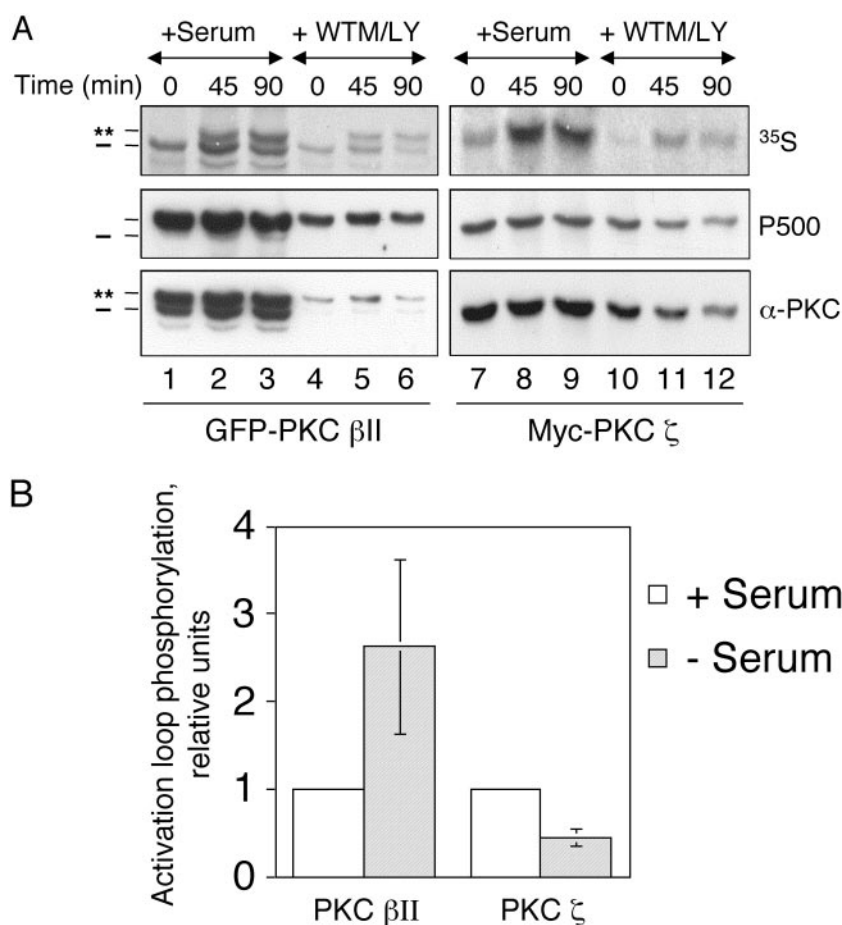
Regulation of Protein Kinase C by PDK-1

FIG. 6. **The maturation of PKC βII is not regulated by PI3-kinase.** NIH 3T3 cells were transiently transfected with GFP-tagged PKC βII or Myc-tagged PKC ζ. The transfected cells were maintained in serum-containing media (*lanes 1–3* and *7–9*) or, 18 h post-transfection, serum-starved overnight and then treated with wortmannin (*WTM*) (100 nM) and LY294002 (*LY*) (30 μM) for 20 min at 37 °C (*lanes 4–6* and *10–12*). Cells were then pulsed with [^{35}S]methionine/cysteine for 7 min and chased for 0, 45, and 90 min. The labeled GFP-PKC βII and myc-PKC ζ were immunoprecipitated with anti-PKC α and anti-Myc antibody, respectively. The immunoprecipitated proteins were separated on a SDS-PAGE gel and analyzed by PhosphorImaging and immunoblotting. *A, top panel* shows autoradiograms of ^{35}S-labeled PKC βII and ζ in immunoprecipitates (*i.e.* newly synthesized protein); *middle panel* shows activation loop phosphorylation of PKC detected by a phospho-specific antibody P500 (*i.e.* activation loop phosphorylation in total population of PKC); *bottom panel* shows the total PKC βII (*left*) and ζ (*right*) in the immunoprecipitates detected using the anti-PKC βII or anti-Myc antibody, respectively. Unphosphorylated PKC βII is labeled with a *dash*, and the mature, fully phosphorylated species is labeled with *two asterisks*. *B,* the data in the Western blots shown in *A* were quantified by CCD camera using a GeneGnome bioimaging system (Syngene) analysis to show the amount of activation loop phosphorylation, as detected by the P500 antibody, normalized to the amount of total PKC. *Open bars* represent phosphorylation in the presence of serum, and *hatched bars* represent phosphorylation in the absence of serum. Data are expressed in relative units and represent the ratio of the P500 stain to the stain using the anti-PKC βII or anti-Myc antibody. The relative phosphorylation of PKC βII and ζ under the serum-containing condition was normalized to 1, and the value of the serum-starved cells was compared with that of the serum-treated cells. The relative phosphorylation of PKC $\beta\Theta\Theta$ and ζ from the serum-starved cells was 3 ± 1 (*n* = 3) and 0.4 ± 0.1 (*n* = 3), respectively.

phosphorylated on the activation loop (*A*) and on the hydrophobic phosphorylation motif (*B*). In addition, Western blotting with an antibody that recognizes both phosphorylated and dephosphorylated species of PKC revealed that PKC migrated as a single band in serum-starved (and control) cells (*C*), consistent with quantitative processing (*i.e.* both carboxyl-terminal sites were phosphorylated resulting in only one species of PKC detectable on SDS-PAGE). Quantitation of the degree of activation loop phosphorylation relative to total PKC α revealed that the PKC α in serum-treated cells had an ~2-fold reduction in activation loop phosphorylation relative to the PKC α in cells treated with PI3-kinase inhibitors (Fig. 7*D*). This decrease in activation loop phosphorylation upon serum treatment mirrored the decrease observed for overexpressed PKC βII (Fig. 6*B*).

To confirm that our protocol for inhibiting PI3-kinase was effective, we examined the effect of the above treatments on the phosphorylation of Akt, a PDK-1 substrate whose phosphorylation is dependent on PI3-kinase (17). The pulse-chase exper-

iment in Fig. 8 shows that, in the presence of serum, Akt was processed to a slower migrating species with a half-time on the order of 25 min. In contrast, Akt from cells treated with wortmannin and LY294002 migrated exclusively as the faster migrating species even after 50 min of chase. Analysis with an antibody that recognizes the phosphorylated activation loop of Akt revealed robust phosphorylation of Akt from serum-treated cells. This phosphorylation was absent in wortmannin and LY294002-treated cells. These results establish that our conditions for depleting cells of 3′-phosphoinositides were effective and prevented activation loop phosphorylation of Akt.

DISCUSSION

Pulse-chase analysis of the processing of conventional PKC isozymes reveals that the phosphorylation by PDK-1 occurs preferentially at the membrane, is the first step in the maturation of PKC, and is independent of PI3-kinase. We have previously shown that newly synthesized PKC adopts an "open" conformation in which the pseudosubstrate is excluded
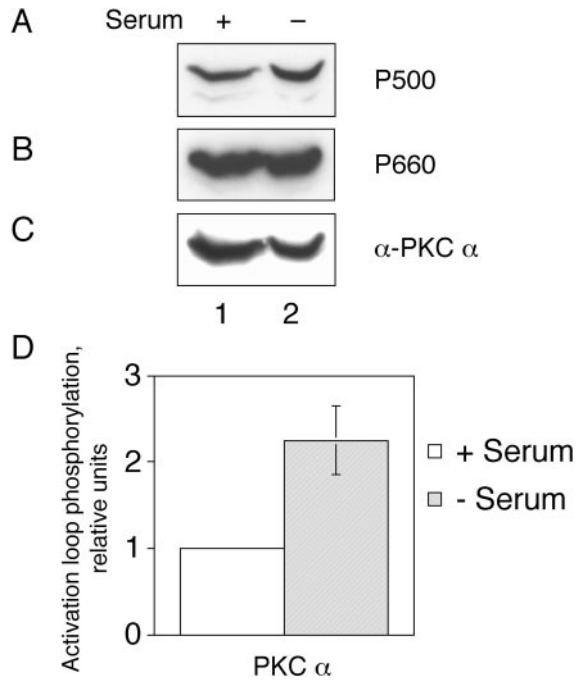
FIG. 7. **The PDK-1-mediated phosphorylation of endogenous PKC α is not regulated by PI3-kinase.** NIH 3T3 cells were maintained in serum-containing growth media (*lane 1*) or serum-starved overnight and then treated with wortmannin (100 nM) and LY294002 (30 μM) for 20 min at 37 °C (*lane 2*). Cells were then lysed in buffer containing 1% Triton X-100, and Triton-soluble fractions were analyzed using SDS-PAGE and immunoblotting. *A,* the activation loop phosphorylation of endogenous PKC α was detected by the phospho-specific antibody P500. *B,* the hydrophobic site phosphorylation of endogenous PKC α was detected by the phospho-specific antibody P660. *C,* total protein expression of PKC α was detected by the anti-PKC α polyclonal antibody. *D,* the signals in the Western blots shown in *A* and *C* were quantified by CCD camera using a GeneGnome bioimaging system (Syngene). The amount of activation loop phosphorylation as detected by the P500 antibody was normalized to the amount of total protein, and this value is termed relative phosphorylation. The relative phosphorylation of endogenous PKC α under the serum-containing condition was normalized to 1, and the value of the serum-starved cells was compared with that of the serum-treated cells. The *open bar* represents phosphorylation in the presence of serum, and the *hatched bar* represents phosphorylation in the absence of serum. The relative phosphorylation of the serum-starved cells was 2.3 ± 0.4 (*n* = 2).

FIG. 8. **The serum-dependent phosphorylation of Akt is abolished by PI3-kinase inhibitors.** NIH 3T3 cells were transiently transfected with HA-tagged Akt. The transfected cells were maintained in serum-containing media (*lanes 1–3*) or, 18 h post-transfection, serum-starved overnight and then treated with wortmannin (*WTM*) (100 nM) and LY294002 (*LY*) (30 μM) for 20 min at 37 °C (*lanes 4–6*). The cells were then pulse-labeled with [^{35}S]methionine/cysteine for 7 min and chased for 0, 25, and 50 min. Akt was immunoprecipitated with the anti-HA antibody, and immunoprecipitated proteins were separated on SDS-PAGE and subjected to PhosphorImager analysis and immunoblotting. The *top panel* shows the autoradiograph of immunoprecipitated ^{35}S-labeled HA-Akt; the *middle panel* shows the activation loop phosphorylation of Akt detected by a phospho-specific antibody, P308; the *bottom panel* shows the total amount of HA-Akt in immunoprecipitates detected using the anti-HA antibody. The *two open arrows* indicate the two species of Akt observed in serum-treated cells; only the faster-migrating species is present in wortmannin and LY294002-treated cells.

from the substrate-binding cavity, thus exposing the activation loop for phosphorylation by PDK-1 (20). We have also shown that PDK-1 forms a complex with unphosphorylated PKC by binding its exposed carboxyl terminus (26). Taken together with data from this study, our results converge upon a model in which PDK-1 binds newly synthesized PKC by a mechanism that does not require binding of 3′-phosphoinositides to the PH domain of PDK-1. Rather, PDK-1 is constitutively active, and its phosphorylation of PKC is regulated by its direct binding to the open conformation of membrane-localized PKC.

Newly Synthesized Protein Kinase C Associates with a Detergent-soluble Fraction—Pulse-chase analysis of endogenous PKC α in COS-7 cells reveals that newly synthesized enzyme preferentially associates with a Triton X-100-solubilizable membrane fraction, where it is processed by phosphorylation with a half-time of ~5–10 min. This processing can be detected by the change in electrophoretic mobility that accompanies phosphorylation of the two carboxyl-terminal sites, the turn motif and the hydrophobic motif. We have shown previously (8) that phosphate at each of these positions contributes to a change in electrophoretic mobility corresponding to ~2 kDa, with phosphorylation at both positions causing the electrophoretic mobility of PKC to be retarded by the equivalent of 4
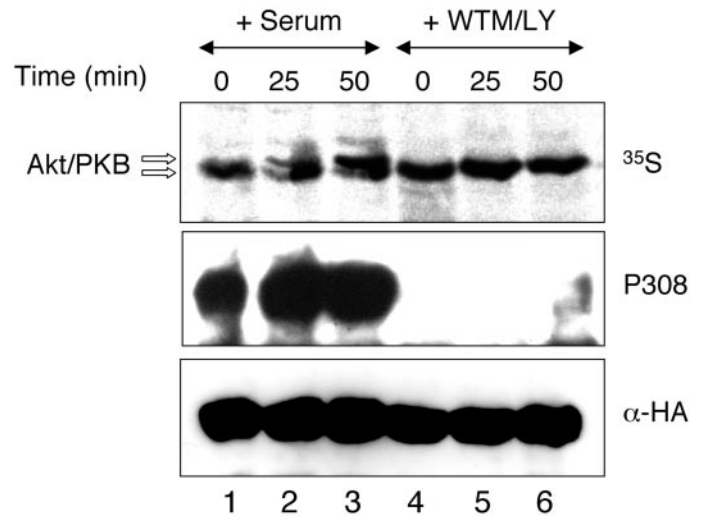
kDa. Intermediate migrating species are not readily detected in our pulse-chase analysis, even with time points taken every 5 min (data not shown), indicating that the two-carboxyl phosphorylations are tightly coupled. Western blot analysis of the total pool of PKC reveals that once phosphorylated, the mature form preferentially partitions in the cytosol.

The exact nature of the membrane fraction where newly synthesized protein kinase C associates is not known. One possibility is the endoplasmic reticulum. However, endoplasmic reticulum-associated proteins retain significant detergent insolubility (27), suggesting that this is not the membrane fraction where protein kinase C becomes processed by phosphorylation. Whether the membrane fraction represents the plasma membrane remains to be elucidated.

Western blot analysis of PKC in subcellular fractions has previously revealed that unphosphorylated wild-type PKC and non-phosphorylatable mutants associate with the Triton X-100-insoluble fraction of cells (8). This led us to hypothesize that PKC associates with the Triton X-100-insoluble fraction of cells before it is phosphorylated and is released into the cytosol following phosphorylation. Indeed, early pulse-chase experiments with PKC α supported such a model; Fabbro and co-workers (28) presented evidence suggesting that newly synthesized PKC α first accumulates in the SDS-soluble fraction of human breast cancer MDA-MB-231 cells before associating with the Triton-soluble fraction. However, the pulse-chase experiments in this study reveal that this is not the case; there is no significant accumulation of newly synthesized PKC in the SDS-soluble (and Triton X-100-insoluble) fraction of COS-7 cells. Rather, newly synthesized PKC preferentially associates with a Triton X-100-soluble membrane fraction. Even phosphorylation site mutants such as T500AAA, which accumulate in

the Triton X-100-insoluble fraction, are associated with the Triton-soluble fraction following their biosynthesis. Parker and co-workers (29) have also found that phosphorylation site mutants of PKC α partition in the detergent-soluble fraction of cells following their biosynthesis. Thus, the unphosphorylated PKC that accumulates in the detergent-insoluble fraction likely reflects mature PKC that has been dephosphorylated. In this regard, phorbol ester treatment of cells promotes the dephosphorylation of PKC, an event that is accompanied by accumulation of the enzyme in the detergent-insoluble fraction (22, 30).

Kinase-inactive and phosphorylation site mutants of PKC accumulate in the detergent-insoluble fraction of cells (22, 23), yet, as this study reveals, constructs such as T500AAA associate with the membrane following their biosynthesis. One possibility is that unphosphorylated species of PKC are not as stable as the phosphorylated species. Thus, persistence of unphosphorylated species may result in aggregation and insolubility. Consistent with this, lack of negative charge at the two carboxyl-terminal positions decreases the stability of the enzyme (22, 23, 29, 31, 32). Thus, the PKC in the detergent-insoluble fraction in overexpression studies may reflect accumulation of aggregated protein that did not get processed quickly enough by PDK-1.

The First Step in Protein Kinase C Processing Is Activation Loop Phosphorylation but the Second Step, Release of PDK-1 and Autophosphorylation, Is Rate-limiting—The first step in the maturation of PKC is phosphorylation by PDK-1; mutation of the activation loop site (*e.g.* T500AAA) prevents phosphorylation of the carboxyl-terminal sites. Several lines of evidence suggest that this phosphorylation is not the rate-limiting step in the maturation of PKC, but rather the subsequent autophosphorylation may limit the rate of processing of PKC. First, mutation of Thr-641 to Glu doubled the rate of processing of PKC, suggesting that phosphorylation of this site is limiting. In contrast, phosphorylation of Ser-660 is not rate-limiting as mutation of this residue to Glu did not accelerate the maturation of PKC. This is consistent with the order of phosphorylations; autophosphorylation of Ser-660 follows that of the rate-limiting Thr-641 autophosphorylation. Second, overexpression of PDK-1 slows the rate of processing. This is consistent with the finding that docking of PDK-1 on the carboxyl terminus blocks autophosphorylation; higher levels of PDK-1 would make it less likely for release of PDK-1 and less likely for the rate-limiting autophosphorylation to proceed. Conversely, we have shown previously (26) that disruption of the docking of PDK-1 on PKC promotes the autophosphorylation of PKC. Taken together, these data suggest that PDK-1 docks onto PKC, phosphorylates the activation loop, and that the release of PDK-1 is rate-limiting and dictates the rate of autophosphorylation and hence maturation.

Curiously, mutation of the hydrophobic site to Ala slowed the maturation of PKC βII. Parker and co-workers (29) reported a similar effect of mutating the hydrophobic phosphorylation site in PKC α to Ala, leading them to suggest that phosphorylation of the hydrophobic motif is the rate-limiting step for phosphorylation of PKC. The recent finding that the hydrophobic phosphorylation motif is a critical determinant for the docking of PDK-1 to its substrates (26, 33) unveils an alternate explanation. Specifically, co-immunoprecipitation studies show that the S660A mutant has a reduced interaction with PDK-1 compared with wild-type enzyme (26). Thus, it is likely that mutation of the hydrophobic site impairs the binding of PDK-1 to PKC, decreasing the rate of phosphorylation. Consistent with this, mutation of the hydrophobic mutation in PKC ζ and PKC-related kinase 2 was shown to impair binding to PDK-1 and

consequent activation loop phosphorylation of these kinases (33).

Our pulse-chase analysis revealed that intermediate phosphorylation species (*i.e.* phosphorylated at the turn motif (Thr-641 in PKC βII) but not the hydrophobic motif (Ser-660 in PKC βII)) do not accumulate during the maturation of PKC. This is consistent with the finding that the rate of processing is limited by phosphorylation of Thr-641 but not Ser-660. The tight coupling of turn motif phosphorylation to that of the hydrophobic site likely reflects the intramolecular mechanism of autophosphorylation at the hydrophobic site (14).

Membrane Binding of PDK-1 Is Not Required for Phosphorylation of Protein Kinase C—The presence of a PH domain in PDK-1 opens the possibility that membrane interactions regulate the function of this kinase. Indeed, *in vivo* studies reveal that the PH domain of PDK-1 binds 3′-phosphoinositides with higher affinity than the archetypal 3′-phosphoinositide-regulated kinase, Akt (15, 16). In support of this, *in vivo* studies suggest that mitogen treatment causes PDK-1 to translocate from the cytosol, where it accumulates in serum-starved cells, to the plasma membrane (19, 34).

To test the role of the PH domain in the processing of PKC, we examined the rate of maturation of PKC βII in cells overexpressing wild-type PDK-1 or a construct deleted in the PH domain. Protein kinase C βII was efficiently processed in cells expressing either construct. In fact, deletion of the PH domain accelerated the rate of processing ~4-fold. Thus, engagement of the PH domain on membranes is not required for PDK-1 to phosphorylate PKC. The accelerated processing of PDK-1 resulting from deletion of the PH domain suggests that this module serves a modest auto-inhibitory role. This is consistent with a recent study showing that removal of the PH domain of PDK-1 increases the phosphorylation of constructs of Akt, also deleted in the PH domain, both *in vitro* (16) and *in vivo* (19). Further support for an autoinhibitory role of the PH domain comes from our finding that deletion of the PH domain strengthens the interaction of PKC with PDK-1.[3] Importantly, our data reveal that the PH domain is not required for the processing of PKC.

The Phosphorylation of Conventional Protein Kinase Cs by PDK-1 Is Independent of PI3-Kinase—Consistent with the finding that the PH domain is not required for the phosphorylation of PKC by PDK-1, we found that the processing of conventional PKCs is independent of PI3-kinase. Specifically, the rate of maturation of PKC βII was comparable in NIH 3T3 cells treated with serum or treated with the PI3-kinase inhibitors wortmannin and LY294002. This was not a result of examining overexpressed enzyme; endogenous PKC α was quantitatively processed by phosphorylation in inhibitor-treated cells, as revealed by its electrophoretic mobility, its phosphorylation on the hydrophobic motif, and its activation loop phosphorylation. In marked contrast, activation loop phosphorylation of Akt was abolished in cells treated with the PI3-kinase inhibitors.

In contrast to the conventional PKCs, the atypical PKC ζ displayed moderate sensitivity to PI3-kinase inhibitors. Specifically, treatment of NIH 3T3 cells with wortmannin and LY294002 reduced the activation loop phosphorylation of PKC ζ ~3-fold. Several studies support the finding that the activation loop phosphorylation of PKC ζ is under moderate, but not absolute, control by PI3-kinase. For example, platelet-derived growth factor treatment of serum-starved NIH 3T3 cells was shown to triple the activity of PKC ζ, presumably as a result of activation loop phosphorylation (11). In addition, LY294002 treatment of serum-starved 293 cells was reported to reduce

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[3] T. Gao and A. C. Newton, unpublished data.

the basal activation loop phosphorylation of PKC ζ (12). Similarly, insulin treatment of adipocytes has been reported to cause a 2-fold increase in activation loop phosphorylation of PKC ζ (35). By using pseudosubstrate deletion mutants, Farese and co-workers (35) have obtained evidence that at least some of this PI3-kinase dependence arises from allosteric control of PKC ζ by 3′-phosphoinositides, rather than by direct effects of these lipids on PDK-1 activity; this isozyme has, indeed, been reported to be directly activated by 3′-phosphoinositides *in vivo* (36). However, regulation of PDK-1 itself by 3′-phosphoinositides may modulate activation loop phosphorylation of PKC ζ because sensitivity of this phosphorylation to PI3-kinase inhibitors is lost upon deletion of the PH domain of PDK-1 (12).

A recent report (37) has identified a PDK-1 isozyme in *Caenorhabditis elegans*, PIAK, that does not have a PH domain and is not phospholipid-dependent. The possibility that such an isoform exists in mammalian cells and could be the predominant isoform to regulate the PKCs is a possibility that needs to be entertained based on our current findings. However, the levels of PKC isoforms are dramatically reduced in PDK-1 $-/-$ embryonic stem cells (38). This would suggest that PDK-1 is the relevant kinase for the maturation of the PKCs, with lack of phosphorylation resulting in accumulation of unphosphorylated PKC in the detergent-insoluble fraction and subsequent degradation. Furthermore, overexpression of catalytically inactive PDK-1 results in a decrease in the fraction of mature PKC in COS-7 cells (10). Thus, data from this and other studies are consistent with a constitutive processing of conventional PKC by PDK-1.

Dephosphorylation of the Activation Loop Is Serum-sensitive—A surprising finding from our study was that serum stimulation decreased the activation loop phosphorylation of both endogenous PKC α and overexpressed PKC βII. This decreased activation loop phosphorylation did not reflect a change in the rate of phosphorylation because pulse-chase analysis, discussed above, established that PKC is processed at the same rate in the presence or absence of serum. Thus, the altered phosphorylation must reflect altered dephosphorylation kinetics. We have shown previously (8) that the PKC in the detergent-soluble fraction of both cultured cells and tissue is quantitatively phosphorylated at the two carboxyl-terminal sites but only about half the population of this mature enzyme is phosphorylated at the activation loop. Thus, following its maturation, the activation loop becomes a specific phosphatase target. The data in this study reveal that this dephosphorylation is stimulated by serum. Whether this serum dependence reflects direct modulation of activation loop phosphatase activity by PI3-kinase lipid products or whether it reflects control by these lipids of activation loop accessibility remains to be explored.

Whereas the dephosphorylation of the activation loop is stimulated by serum, a number of studies suggest that the dephosphorylation of the hydrophobic site is inhibited by serum and stimulated by serum deprivation (39, 40). Thus, the phosphorylation state of PKC is under complex regulation by pathways that are either not sensitive to serum (*e.g.* activation loop phosphorylation by PDK-1 for conventional but not atypical PKCs), phosphatase activity that is serum-stimulated (activation loop), and phosphatase activity that is serum-inhibited (hydrophobic site).

Conclusions—This study supports a model in which PDK-1 is constitutively active in cells, with phosphorylation of its downstream targets being regulated by the conformation of the substrate. In the case of PKC, newly synthesized enzyme is in a conformation that promotes binding of PDK-1 to its carboxyl terminus and in which the activation loop is exposed for phosphorylation. Thus, the primary regulation of this phosphorylation event is the direct interaction of PKC with PDK-1. Although the physiologically relevant location for the maturation of PKC is at the membrane, the phosphorylation of PKC by PDK-1 does not require the PH domain of PDK-1 and is not affected by 3′-phosphoinositides. Thus, despite the name, there is no phosphoinositide dependence to the phosphorylation of conventional PKCs by PDK-1.

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