Regulation of Protein Kinase D by multisite phosphorylation.

Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis

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RUNNING TITLE

In vitro and in vivo PKD phosphorylation sites
SUMMARY

Activation of the serine/threonine kinase, protein kinase D (PKD/PKCµ) via a phorbol ester/PKC-dependent pathway involves phosphorylation events. The present study identifies five *in vivo* phosphorylation sites by mass spectrometry, and the role of four of them was investigated by site-directed mutagenesis. Four sites are autophosphorylation sites, the first of which (Ser-916) is located in the carboxyl terminus: its phosphorylation modifies the conformation of the kinase and influences duration of kinase activation, but is not required for phorbol ester-mediated activation of PKD. The second autophosphorylation site (Ser-203) lies in that region of the regulatory domain, which in PKCµ interacts with 14-3-3τ. The last two autophosphorylation sites (Ser-744 and Ser-748) are located in the activation loop, but are only phosphorylated in the isolated PKD-catalytic domain and not in the full-length PKD; they may affect enzyme catalysis, but are not involved in the activation of wild-type PKD by phorbol ester. We also present evidence for proteolytic activation of PKD. The fifth site (Ser-255) is transphosphorylated downstream of a PKC-dependent pathway after *in vivo* stimulation with phorbol ester. *In vivo* phorbol ester stimulation of an S255E mutant no longer requires PKC-mediated events. In conclusion, our results show that PKD is a multisite phosphorylated enzyme, and suggest that its phosphorylation may be an intricate process which regulates its biological functions in very distinct ways.
INTRODUCTION

Protein kinase D (PKD) is a serine/threonine protein kinase, also called PKCµ, that was first described as a member of the novel protein kinase C (PKC) subgroup (δ, ε, η and θ) (1, 2). PKD contains two cysteine-rich domains that bind diacylglycerol (DAG) or phorbol ester, but it lacks the calcium binding domain seen in the classical PKCs. However, PKD also contains a pleckstrin homology domain that regulates its kinase activity (3), but does not harbor the typical PKC autoinhibitory pseudosubstrate motif. Moreover, the PKD catalytic domain is only distantly related to the kinase subdomains of the PKC family, but shows homology to that of the Ca²⁺-regulated kinases, such as myosin light chain kinase and calcium/calmodulin kinase I. Finally, the substrate specificity of PKD is probably different from that of other PKCs, since it is specific for a unique peptide sequence (4). These characteristics have rendered it difficult to classify PKD in the scheme of protein kinases (5) and PKD might be the first member of a new protein kinase family and/or subgroup.

Regulation of protein kinases is achieved through a variety of mechanisms that include auto- and transphosphorylation events and control by regulatory domains or subunits. Mutagenesis studies have highlighted the regulatory domain of PKD/PKCµ in the negative control of its activity (3, 6). Despite the fact that most protein kinases share a largely conserved catalytic domain structure, their regulation by phosphorylation is very diverse (7, 8). Phosphorylation of specific threonine, serine or tyrosine residues can occur at a number of sites, some of which are located at the N-terminal or C-terminal ends of the enzyme (e.g. in calmodulin-dependent kinase II and PKCβII) or on other subunits (e.g. on phosphorylase kinase). A key feature for regulation is the phosphorylation of residues in the so-called kinase “activation loop” located between subdomains VII and VIII of the kinase core. Here again, this general mechanism holds for most, but not for all protein kinases. Phosphorylation of residue(s) in the activation loop may be due to autophosphorylation (e.g. cAPK², c-Src and IRK) or transphosphorylation catalyzed by another protein kinase (e.g. PKBα, p70S6K, ERK, PKCζ). Finally, the phosphorylation of protein kinases, either by autophosphorylation or transphosphorylation, can be the cause of activation or its consequence. For example, three phosphorylation events regulate PKCβII activation, the first is catalyzed by an upstream
kinase (probably PDK-1), which phosphorylates Thr-500 in the activation loop thereby leading to kinase activation and autophosphorylation of two other sites in the C-terminus (9).

PKD/PKCµ has been shown to be activated by pharmacological agents such as phorbol ester and bryostatin 1 (10, 11, 12) and by physiological stimuli such as platelet-derived growth factor (PDGF), tumor-necrosis-factor (TNF), angiotensin II and neuropeptide agonists (13, 14, 15, 16). Recent data have shown that PKD plays a role in the regulation of Golgi structure and function (17). Interestingly, PKD may also serve as a molecular switch to promote cell proliferation, while inhibiting apoptosis (16, 18, 19). PKD activation was first described as a phorbol ester or diacylglycerol/phospholipid-dependent process. In vitro and in vivo experiments have shown that immunopurified PKD is markedly stimulated by either biologically active phorbol ester or diacylglycerol, in the presence of phosphatidylycerine (10, 11, 20). More recently, attention was focussed on phosphorylation events that control the PKD activity (21). These observations were based on the fact that PKD activation was maintained during cell disruption and immunoprecipitation. Additional data, including the use of PKC inhibitors and cotransfection of PKD with constitutively active mutants of PKCε and PKCη, indicated that PKD was activated by phosphorylation in vivo through a PKC-dependent signal transduction pathway (11, 12, 13). Recent results have demonstrated that PKCη interacts with the PH domain of PKD, suggesting a direct link between PKCη and PKD (22).

Little is known about how phosphorylation regulates PKD activity, and the phosphorylation sites that mediate its biological functions have not been identified. The group of Rozengurt proposed that the in vivo activation of PKD by phorbol ester results from the phosphorylation of two activation loop serine residues, namely Ser-744 and Ser-748, via a novel PKC-dependent signal transduction pathway (23). However, no sequence studies were undertaken to unambiguously determine that these two serines were actually being phosphorylated in vivo. The C-terminal Ser-916 was suggested to be autophosphorylated in PKCµ/PKD, as it was recognized by a phosphospecific peptide antibody (24). Phosphorylation of Ser-916 was also reported to be induced by phorbol ester treatment of cells in vivo.
The present study identifies five phosphorylation sites in PKD by mass spectrometry and several of these sites were individually mutated to alanine or glutamate to study their functional role.
EXPERIMENTAL PROCEDURES

Materials

DMEM and phosphate-free DMEM were from Life Technologies. Protein A-TSK gel was from Affiland (Sart-Tilman, Belgium). Glutathione Sepharose 4B was from Pharmacia (Upsala, Sweden). Sequencing grade trypsin and chymotrypsin were from Boehringer. HPLC solvents were from Lab Scan. Bisindolylmaleimide I (Gö 6850) was from Calbiochem. Shrimp alkaline phosphatase, [γ-32P]ATP and 32P orthophosphate were from Amersham. All other materials were from Sigma or Boehringer.

Site-directed mutagenesis, expression and purification

The phagemid, called pBluescript (SK)II+/PKD (pBS/PKD), containing the full-length PKD cDNA (10), was used as a template to create eight single mutations (S916A, S916E, S744A, S748A, S744E, S748E, S255A and S255E) using the Quickchange kit (Stratagene) following instructions provided by the manufacturer. The different mutations were verified by restriction analysis and DNA sequencing. A kinase-dead mutant of PKD (K628N) was also generated by the same strategy.

The DNA sequence encoding wild-type or mutated PKD was subcloned into the eukaryotic expression vector pGMEX-T3 that has been used to overexpress Gluthatione S-transferase (GST)-fusion proteins in eukaryotic cells under an EF1a promoter. The pBS/PKD phagemids were cleaved with Not I to release the cDNA for PKD, which was then inserted into the compatible ends of pGMEX-T3 to create pGMEX-T3-PKD. To overexpress untagged-PKD constructs, wild-type and mutant proteins were also cloned in pcDNA3 vector as described (10).

To prepare the PKD catalytic domain fusion protein (GST-catPKD), a 1014-base pair fragment comprising the entire catalytic domain of PKD was generated by PCR and inserted into pBluescript (SK)II+. The assembled fragment was then subcloned into pGMEXT-3 between the Sall and NotI restriction sites.
To prepare purified GST-PKD or GST-catPKD, 10 cm diameter dishes of human embryonic kidney 293 T cells (HEK 293T), expressing the SV40 large T antigen, were cultured and each dish transfected with 7 µg of pGMEX-T3-PKD plasmid DNA using the modified calcium phosphate method (25). Briefly, 2×10^6 HEK 293T cells per dish were grown for 24 hours before transfection at 37°C and 5% CO₂ in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The DNA was mixed with equal volumes of 0.25M CaCl₂ and BES-buffered solution and incubated for 30 min at room temperature. The calcium phosphate-DNA solution was added onto medium-containing plates and incubated for 16 hours at 37°C, 3% CO₂. The medium was then replaced with fresh DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin. The cells were used for experimental purposes 48 hours later. Phorbol ester treatment was with 1µM PDBu for 15 min. The cells were then washed once with ice-cold PBS and each dish lysed in 1 ml of ice-cold buffer A, pH 7.5 (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 50 mM NaF, 5 mM NaPPi, 0.2 µM microcystin, 0.27 M sucrose, 1 mM AEBSF, 1 µg/ml Leupeptin, 15 mM β-mercaptoethanol, 1% (v/v) Triton X-100). Lysates were briefly vortexed and centrifuged at 1000 g for 15 min. The supernatants were pooled and incubated for 1 hour at 4°C on a rotating platform with 25 µl (gel bed vol.) per dish of Glutathione-Sepharose previously equilibrated in one bed volume of Buffer A. The suspension was centrifuged for 10 min at 3000 g and the beads were washed once with 10 bed vol. of Buffer A containing 0.5 M NaCl and once with 10 bed vol. of Buffer B, pH 8.0 (50 mM Tris, 0.1 mM EGTA, 0.27 M sucrose, 15 mM β-mercaptoethanol, 10 % glycerol (v/v), 50 mM NaCl). GST-PKD was eluted from the gel with 2 times one bed volume of Buffer B containing 20 mM reduced glutathione. The combined eluates were divided into aliquots and stored at -80°C.

**PKD kinase assay and immunoprecipitation**

PKD activity was measured with syntide-2 peptide as substrate (1) under the conditions described in the legends to the figures and tables. One Unit of PKD activity corresponds to the amount of enzyme catalysing the formation of 1 nmol of product per min
under the assay conditions. Immunoprecipitation of PKD from cell lysates with a polyclonal antibody and kinase assay was as described (15).

**In vitro autophosphorylation assay**

Purified GST-PKD (30 µg) was incubated in buffer containing 15 mM Tris, pH 8.0, 5 mM MgCl₂ and 0.1 mM [γ-32P] MgATP (specific radioactivity of 200 cpm/pmole) for up to 60 minutes at 30°C. The reaction was stopped by adding SDS-PAGE sample buffer (1% SDS, 10% glycerol, 50 mM dithiotreitol, 12 mM Tris-HCl, pH 6.8) and the samples were boiled for 5 min for SDS-PAGE in 7.5% acrylamide gels. To determine 32P incorporation, gels were stained with Coomassie Blue. The bands corresponding to GST-PKD were counted in a Hewlett-Packard Instant Imager together with spotted dried aliquots of the diluted stock solution of [γ-32P]MgATP used in the phosphorylation experiments. Stoichiometries of 32P incorporation (mol/mol of enzyme) were calculated from the amount of protein loaded onto the gel as quantified by the ninhydrin method (see below), and the molecular weights of the GST-PKD and GST-catPKD, taken as 127575 Da and 66975 Da, respectively.

**In vivo labeling**

HEK 293T cells were cultured and transfected as described above. The dishes were washed 5 times with phosphate free DMEM containing 100 units/ml penicillin and 100 µg/ml streptomycin and labeled for 4 hours with 3 ml per dish of phosphate free DMEM containing 150 µCi/ml of 32P orthophosphate. Cells were stimulated with PBDu and the PKD-GST proteins purified as described above.

**Identification of Phosphorylation sites by Electrospray Ionization-tandem Mass Spectrometry (ESI-MS/MS)**

To identify autophosphorylation sites, GST-PKD (50 µg) was incubated at 30°C, with 0.1 mM [γ-32P] MgATP (specific activity 300-500 cpm/pmol). After 60 min, the reaction was stopped by adding 10% trichloroacetic acid (v/v) and left on ice for one hour. Precipitated protein was collected by centrifugation (12000 g × 15 min), washed once with ice-cold acetone and resuspended in 50 µl 0.1M Tris/HCl pH 8.5, 0.6% (w/v) n-octylglucoside for overnight digestion at 30°C with 1 µg of sequencing grade chymotrypsin or trypsin. Peptides were
separated by reversed-phase narrowbore HPLC on a Vydac C18 column (1.0 mm×25 cm) in an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (solvent A). Elution was performed with the following gradient program: 5-100% solvent B (70% acetonitrile in solvent A) over 100 min at a flow rate of 40 µl/min generated by a model 140B Applied Biosystems solvent delivery system. Peptides were collected by hand and radioactive peptides were identified by Cerenkov counting. Radioactive peptides were dried under vacuum and redissolved in 4-6 µl of 60% (v/v) methanol, 1% (v/v) acetic acid for nanospray ESI-MS/MS.

To identify transphosphorylation sites, GST-PKD (50 µg) purified from ³²P-labeled cells, was precipitated, redissolved and digested as described above. Peptides were separated by reversed-phase HPLC on a Pharmacia C2/C18 column (2.1 mm×10 cm) connected to a Pharmacia SMART system. Column was equilibrated in 0.1% (v/v) trifluoroacetic acid (solvent A). Elution was performed with the following gradient program: 7-70% solvent B (100% acetonitrile, 0.1% (v/v) trifluoroacetic acid) over 80 min at a flow rate of 80 µl/min. Peptides absorbing at 215 nm were collected, counted, dried and dissolved as described above for nanospray ESI-MS/MS.

Radioactive peaks were analyzed by nanospray ESI-MS/MS. Briefly, 2 to 3 µl of the radioactive peptides were analyzed in a LCQ (Finnigan MAT LCQ, San Jose, CA) equipped with a nano-electrospray ionization source. Spectra were taken in full MS and zoom scan mode to determine parent masses and their charge state. The source voltage was set at 0.8 kV with a scan time of 3.6 seconds. The collision energy was adjusted to the minimum needed for fragmentation.

Identification of Phosphorylation sites by High Performance Liquid Chromatography-Electrospray Ionization- tandem Mass Spectrometry (HPLC-ESI-MS/MS)

To identify in vivo phosphorylation sites, peptides were separated by reversed-phase HPLC on a C18 capillary column (0.3mm×25cm, LC Packings) with an acetonitrile gradient in 0.05% (v/v) formic acid (solvent A). Elution was performed with the following gradient: 0-100% solvent B (95% acetonitrile (v/v) in 0.05% (v/v) formic acid) over 100 min at a flow rate of 5 µl/min generated by a 140B pump (Applied Biosystems) connected to a flow splitter (1/20, Accurate solvent splitter, LC Packings). Mass spectra were recorded on-line in the LCQ.
(Finnigan MAT LCQ, San Jose, CA) using the standard electrospray ionization source. Electrospray was performed at a voltage of 5.6 kV with a scan time of 1.2 seconds. Mass spectra were acquired in a mode that alternated single MS scans (m/z 500-2000) with MS² and MS³ scans.

Other Methods

Protein was measured by the Bradford method (26) using γ-globulin as a standard or by the reaction with ninhydrin after trichloroacetic acid precipitation and complete alkaline hydrolysis (27) using bovine serum albumine as a standard. SDS-polyacrylamide gel electrophoresis analysis in 10% or 7.5% (w/v) acrylamide was as described (28). Kinetic constants were calculated by fitting data to a hyperbola by nonlinear least square regression using a computer program (Ultrafit, Biosoft, Cambridge, UK)
RESULTS

Purification of wild-type and mutant PKD preparations

Engineering of a N-terminal GST-tag in PKD allowed rapid purification of the protein by a one-step procedure. Cell lysates were directly mixed with Glutathione Sepharose 4B and GST-PKD preparations were eluted with reduced glutathione. SDS-polyacrylamide gel electrophoresis analysis of the purified preparations of wild-type PKD (GST-PKD), PKD catalytic domain (GST-catPKD) and mutant proteins showed single 132000-Mr or 68000-Mr bands in agreement with the calculated masses. The purified proteins were stored in elution buffer at -80°C with no appreciable loss of activity over several months.

Characterization of purified wild-type and catalytic domain PKD

The catalytic domain of PKD had a 12-fold higher k_{cat} than wild-type PKD (6 s^{-1} versus 0.5 s^{-1}). The affinities for MgATP and syntide-2 were similar for the two recombinant enzymes, suggesting that the overall structure of the catalytic domain was maintained and that the GST tag had no influence on the kinetic properties of the enzyme (Table 1). Likewise, addition of a N-terminal green fluorescent protein (GFP) tag in PKD has previously been shown to have no influence on PDBu induced translocation, basal catalytic activity, phorbol ester binding and kinase activation (29). Moreover, in vitro incubation of GST-PKD with PS/PDBu micelles led to a 5-fold stimulation of PKD activity (not shown). A comparable stimulation was observed with untagged PKD (10, 23).

Incubation of HEK 293T cells with PDBu caused a 3-fold increase in k_{cat} for GST-PKD with no effect on the affinities for MgATP and syntide-2 (Table 1). By contrast, PDBu treatment had no effect on the activity of GST-catPKD. Therefore, deletion of the regulatory domain of PKD leads to a constitutively active kinase and these results suggest that the region for PKC-dependent activation is located outside the catalytic domain. These results support those obtained in experiments using partial deletions or point mutations in the regulatory domain of PKD (3, 6).
Time course experiments of *in vitro* autophosphorylation showed that $^{32}\text{P}$-incorporation into GST-PKD was maximal after 60 min and was maintained for up to 80 min (not shown). The initial rate of GST-PKD autophosphorylation was independent of enzyme concentration with an activity of 50 pmol/min/mg (not shown), indicating that autophosphorylation of GST-PKD occurs via an intramolecular event at a very slow rate. Indeed, for *Dictyostelium* MLCK, which has a catalytic domain possessing 40% identity with the PKD kinase domain, autophosphorylation is also intramolecular, but with a 15-fold faster rate (30). The stoichiometry of autophosphorylation of GST-PKD and GST-catPKD was 0.4 and 0.2 mole of phosphate incorporated per mol of enzyme, respectively, suggesting the existence of at least two autophosphorylation sites, one located in the catalytic domain, the other in the regulatory domain. The low incorporation of radioactive phosphate *in vitro* could reflect the fact that these sites were already largely phosphorylated *in vivo* (see below).

**Ser-916 is an autophosphorylation site *in vitro* and *in vivo* and is involved in the downregulation of PKD activity after PDBu stimulation.**

Purified GST-PKD was autophosphorylated *in vitro* by incubation with $[\gamma-^{32}\text{P}]\text{MgATP}$. Following TCA precipitation, the protein was digested with chymotrypsin and peptides were separated by reversed phase HPLC, and one major radioactive peak was observed (not shown). This peak was analyzed by nanospray ESI-MS/MS to identify the phosphorylation site. The fraction contained several ions in full MS mode, only one of which ($m/z = 883.4$, P1 in Table 2) lost 98 Da in the ion-trap when subjected to a low collision energy, and its mass was decreased to $m/z = 785.3$ (Fig.1). The difference of 98 Da corresponds to the loss of $\text{H}_3\text{PO}_4$ through beta-elimination, leaving dehydroalanine in place of the phosphorylated serine residue (31). An ion of $m/z = 803.4$ (883.4 minus 80 Da for the $\text{PO}_3^{2-}$ group) could correspond to a theoretical chymotryptic peptide with an average mass of 803.9 Da representing the sequence 912-SERVSI-L-918. Indeed, when the ion of $m/z = 785.3$ was fragmented in the ion-trap, the sequence of the chymotryptic fragment was confirmed and the phosphorylated residue was identified as Ser-916 (Fig1). To see whether this site was phosphorylated *in vivo*, GST-PKD was purified from unstimulated or PDBu stimulated cells and digested with chymotrypsin. The resulting peptides were analysed by on-line capillary
HPLC-ESI-MS/MS. Ser-916 was found to be phosphorylated in both conditions, indicating that Ser-916 is phosphorylated \textit{in vivo} (Table 2). Ser-916 was also found to be autophosphorylated in GST-catPKD, indicating that autophosphorylation of Ser-916 does not depend on the presence of the regulatory domain. Some minor radioactive HPLC peaks contained phosphopeptides generated by missed cleavages during proteolysis (P3 and P4 in Table 2).

To study the role of Ser-916 autophosphorylation, this residue was mutated to alanine (S916A) or glutamate (S916E) and the kinetic properties of PKD were studied. The two mutants had similar kinetic parameters compared with wild-type GST-PKD, with no drastic changes in $k_{\text{cat}}$ or $K_m$ (Table 1). Moreover, the S916A and S916E mutants could be activated in cells treated with PDBu to the same extent as wild-type GST-PKD (Table 1). Mutation of Ser-916 to glutamate did not overcome the need for PKC-activity in the PDBu-mediated PKD activation (see below and Fig.6). Therefore, autophosphorylation of Ser-916 is not required either for activity or for \textit{in vivo} activation by PDBu.

Other roles for phosphorylation sites in the carboxyl terminus in PKCs have been proposed. For example, C-terminal phosphorylation sites may increase protein stability or increase the resistance to dephosphorylation by protein phosphatases (32). C-terminal phosphorylations have also been reported to affect protein subcellular partitioning, sensitivity to proteolysis or affinity for substrates, phosphatidylserine or Ca$^{2+}$ (33, 9). Experiments were therefore undertaken to see whether phosphorylation at Ser-916 in GST-PKD could cause similar changes. For the S916E and S916A mutants, the PS/PDBu dependence of GST-PKD substrate phosphorylation was measured in the presence of mixed micelles containing Triton X-100 (34). None of the mutations significantly affected the PS/PDBu dependence of the GST-PKD activity (not shown). We then tested the sensitivity of PKD towards proteolysis by trypsin. This technique has been used to study conformational changes in PKC, for example those induced by membrane binding (35). Incubation of the \textit{in vivo} PDBu-stimulated wild-type and S916E preparations with trypsin (0.02 units ml$^{-1}$) led to extensive proteolysis of the native enzyme and the appearance of a Mr 42000 fragment, which corresponds to the Mr of the catalytic domain. No intact GST-PKD was left after incubation with 0.2 units ml$^{-1}$ of trypsin. By contrast, the S916A mutant was more resistant to proteolysis requiring higher
concentrations of trypsin to obtain a similar pattern of proteolysis and intact enzyme was still apparent after incubation with 0.2 units ml\(^{-1}\) trypsin (Fig. 2). We also investigated the sensitivity of the \textit{in vivo} PDBu-stimulated wild-type and Ser-916 mutants towards dephosphorylation by alkaline phosphatase. Following \textit{in vitro} autophosphorylation with \([\gamma-^{32}\text{P}]\text{MgATP}\) and incubation with alkaline phosphatase, the wild-type and S916E preparations showed a time-dependent decrease in their extent of phosphorylation, whereas the S916A mutant was resistant to dephosphorylation (Fig. 3). Finally, we investigated whether Ser-916 mutation could affect any \textit{in vivo} properties of PKD, by examining the time dependent downregulation of PKD activity after PDBu stimulation. For this experiment untagged PKD constructs were cloned in pcDNA 3 vector and transiently transfected in HEK-293T cells. After PDBu treatment, cells were washed and incubated for another 6 hours in DMEM medium without PDBu. PKD activity was measured after immunoprecipitation at different time points. The wild-type and S916E mutant showed a time-dependent decrease in activity reaching 56 or 64 % of initial activity respectively, after six hours (Fig.4). The slow downregulation of the PKD activity seen after phorbol ester treatment confirms previous studies (36). In contrast, PKD activity of the S916A increased during the first hour of incubation and decreased thereafter at a similar rate compared to the wild-type reaching 85% of initial activity after 6 hours (Fig.4). We also tested whether this decrease in activity was reversible. After 6 hours of downregulation, cells were restimulated with PDBu without changing the medium. The PKD activity measured after the second PDBu stimulation was similar to the initial activity (after the first PDBu treatment), indicating that downregulation of PKD activity occurs \textit{via} a reversible mechanism, probably reflecting reversible dephosphorylation of the enzyme (Fig.4).

**Identification of Ser-203 as a second \textit{in vitro} and \textit{in vivo} autophosphorylation site**

The S916A and S916E mutants still autophosphorylate, suggesting the existence of other autophosphorylation sites, which were not detected after chymotryptic cleavage. Therefore, autophosphorylated GST-PKD was digested with trypsin instead of chymotrypsin. One major radioactive peak was isolated by HPLC and analyzed by nanospray ESI-MS/MS. This peak did not contain a peptide corresponding to the predicted short sequence containing...
phosphorylated Ser-916 (VpSIL), which may not have been retained by the C18 column. A double charged ion with m/z = 777.2 was found in the major peak, which lost H₃PO₄ in the ion-trap and its m/z decreased to 728.3. This could correspond to the tryptic peptide 201-RLSNVSLTGTVLR-214 (P5 in Table 2). Fragmentation of the m/z = 728.3 ion confirmed the sequence and identified Ser-203 as the autophosphorylation site (not shown). We also looked for this phosphorylation site in vivo by on-line capillary HPLC-ESI-MS/MS. GST-PKD purified from unstimulated or PDBu stimulated cells was digested by trypsin or chymotrypsin. Ions corresponding to phosphopeptides containing Ser-203 were found in both conditions with both proteases (P5 and P7 in Table 2). Fragmentation of the ions losing H₃PO₄ confirmed that Ser-203 is phosphorylated in vivo (Fig 5).

Identification of Ser-255 as an in vivo transphosphorylation site in PKD

HEK-293T cells, transiently expressing GST-PKD, were labeled with [³²P]orthophosphate and stimulated with PDBu. Following cell lysis, GST-PKD was purified, digested with chymotrypsin and the three major radioactive peaks isolated by HPLC were analyzed by nanospray ESI-MS/MS. Two peaks contained the autophosphorylation sites Ser-916 and Ser-203. The third peak contained a double charged ion with m/z = 753.1, which lost H₃PO₄ to give m/z = 704.0 in the ion-trap of the mass spectrometer. Fragmentation of this ion identified a peptide corresponding to the sequence 247-IGREKRSNSQS-Y-258, in which Ser-255 was the phosphorylated residue (P6 in Table2). This peak was not labeled in GST-PKD purified from unstimulated cells. Ser-255 is located between the two cysteine rich domains, and is conserved in PKCθ. It has basic residues at positions -3 and -4 which have been shown to be important for PKC substrate recognition. The sequence lacks positive residues at position +2 and +3, but basic residues at these positions are not absolutely required for novel PKC family members (such as PKCε and PKCη) (4). However, purified PKCε or PKCη preparations did not phosphorylate GST-PKD in vitro (not shown), suggesting an indirect role of these kinases in the activation of PKD. To test the role of Ser-255 phosphorylation in PKD activation, it was mutated to alanine or glutamate (S255A and S255E). The two mutations decreased the kcat of the enzyme (2-to 4-fold) without affecting the affinities for MgATP or syntide-2 (Table 1). Thus, mutation of Ser-255 into glutamate certainly did not induce a
constitutively active form of PKD. However, PDBu treatment led to a greater degree of activation of the S255E mutant than the wild-type (11- versus 3-fold). Surprisingly, mutation of Ser-255 to alanine did not abolish PDBu-induced activation, indicating that this site is not essential for PKD activation. To test whether this site might be phosphorylated downstream of a PKC-dependent signaling pathway, we studied the PDBu-induced activation of the S225E and S255A mutants in the presence and absence of Gö 6850. This PKC inhibitor prevents the activation of PKD in response to phorbol ester or mitogens (11, 15, 21). Treatment of HEK 293T cells with Gö 6850 significantly decreased the PDBu-induced PKD activation of wild-type and S255A GST-PKD, but had no effect on the activation of the S255E mutant (Fig.6), indicating that this site is indeed phosphorylated by a PKC-dependent pathway upon stimulation with phorbol ester.

**In vivo activation of full-length PKD by phorbol ester does not encompass phosphorylation of the activation loop Ser-744 and Ser-748**

Ser-744 and Ser-748 in the activation loop of PKD have been proposed to become phosphorylated in intact cells in response to PDBu stimulation (23). The authors used a combination of mutational analysis and two-dimensional peptide mapping to demonstrate the potential role of these two residues in the activation of PKD. However, the two phosphorylation sites were not unambiguously identified and a detailed kinetic study of the serine mutants was not performed. We did not find any radioactive peptides containing phosphorylated Ser-744 or Ser-748 in $[^{32}P]$ labeled HEK-293T cells that were transiently expressing GST-PKD, with or without PDBu stimulation. However, this alone does not rule out the *in vivo* phosphorylation of these sites, as they could have been already phosphorylated by PDBu-independent mechanisms involved in the maturation of the enzyme, as already described for conventional PKCs (37). We therefore looked for the Ser-744 and Ser-748 phosphorylation sites in the activation loop of PKD by on-line capillary HPLC-ESI-MS/MS. Unlabeled HEK 293T cells were treated with or without PDBu and GST-PKD was purified for digestion with trypsin or chymotrypsin. No phosphopeptides containing Ser-744 and Ser-748 were detected in PDBu-stimulated or unstimulated cells. Moreover, we were able to identify and sequence the peptides in which the two activation loop serines were non-
phosphorylated. This indicates that these two sites are not phosphorylated in full-length PKD, either in PDBu-stimulated or unstimulated cells.

We also searched for Ser-744 and Ser-748 phosphorylation in GST-catPKD. Following in vitro autophosphorylation with [γ-32P] MgATP, GST-catPKD was digested with chymotrypsin and peptides were separated by HPLC. Radioactive peaks were analyzed by nanospray ESI-MS/MS. In addition to the previously identified phosphorylated Ser-916, we were able to identify and sequence another phosphopeptide in which Ser-748 was phosphorylated (P2 in Table 2), indicating that this is an in vitro autophosphorylation site in the expressed catalytic domain. GST-catPKD was also analysed by on-line capillary HPLC-ESI-MS/MS directly following purification from unstimulated cells. Ser-916 and Ser-748 were phosphorylated and we also identified phosphorylated Ser-744 (P8 in Table 2). This suggests that all three sites are phosphorylated in vivo. None of these phosphorylation sites were detected by on-line capillary HPLC-ESI-MS/MS in a kinase-dead mutant of GST-catPKD (K628N). This indicates that Ser-744, Ser-748 and Ser-916 are in vivo autophosphorylation sites in GST-catPKD. Since only Ser-748 and Ser-916 could be autophosphorylated in vitro, we conclude that Ser-744 is constitutively phosphorylated in vivo under basal conditions.

We also decided to investigate by site-directed mutagenesis the role of Ser-744 and Ser-748 in PKD activation by PDBu. The two serine residues were mutated to Glu or Ala in the full-length GST-PKD to generate four single points mutants (S744A, S744E, S748A and S748E). The mutants were then expressed in HEK 293T cells and purified as described above. We studied the effects of the mutations on kinetic parameters (Table 1). Mutation of Ser-744 or Ser-748 to Ala drastically decreased the kcat by 22- or 8-fold, respectively, and increased the Km for syntide-2. The S744E mutant also displayed a lower kcat (4-fold) and there was a slight decrease in affinity for MgATP (2-fold). Mutation of Ser-748 to glutamate had no effect on the kinetic properties of PKD. Interestingly, all mutants could be activated in cells treated with PDBu. The S744A and S748A mutants were activated to the same extent as the wild-type (3 to 5-fold), whereas the S744E and S748E mutants displayed a 24- or 8-fold activation, respectively. Moreover, the S744E and S748E mutants were sensitive to Gö 6850 induced inhibition of PDBu-mediated PKD activation (Fig.6). These results indicate that neither of the
activation loop serines is involved in PDBu-induced activation, but that they may be involved in catalysis or in maintaining the conformation of the enzyme protein. Phosphorylation of Ser-744 and Ser-748 in GST-catPKD is probably responsible for the higher catalytic activity of this construct. Indeed mutation of Ser-744 to alanine in GST-catPKD decreased the $k_{cat}$ 20-fold to 0.25 s$^{-1}$.

Activation of PKD by proteolysis

PKC was originally described as a protein kinase which could be activated by limited proteolysis (38). However, it is now generally accepted that a reversible activation of PKC, rather than irreversible proteolytic activation, is the major means of regulation for this family of kinases (39). Nevertheless, an increasing number of kinases having a large regulatory domain (PAK, MEKK-1, PKCδ, PRK1) are activated by proteolysis (40, 41, 42, 43). We tested whether PKD could be activated by proteolysis. Purified GST-PKD was incubated with 0.02 units ml$^{-1}$ of trypsin and a time-course of PKD activation was studied (Fig.7). Partial proteolysis resulted in the appearance of three major bands analyzed by SDS-PAGE with $Mr$s of 90000, 42000 and 26000 (Fig.7), and resulted in an increase in the PKD activity (4 to 14-fold depending on the PKD/Trypsin molar ratio). In vivo treatment with PDBu prior to proteolysis had no effect on this proteolytic activation, suggesting that the trypsin cleavage sites do not only become exposed after phorbol ester stimulation (Fig.7).
DISCUSSION

In this study we identified five phosphorylation sites in PKD, shown schematically in Fig. 8. Two sites are located in the regulatory domain (Ser-255 and Ser-203) in a region which not only mediates the interaction of PKD/PKC\(\mu\) with other proteins, but also controls the catalytic activity of the enzyme. Two other sites are present in the activation loop (Ser-744 and Ser-748), but are only phosphorylated by the isolated catalytic domain and not by the full-length protein. Their phosphorylation is not regulated by phorbol ester. The presence of a C-terminal phosphorylation site (Ser-916) links PKD to the growing number of kinases phosphorylated on their carboxyl terminus, an event which is thought to provide an electrostatic anchor that structures the kinase and/or alters its surface to promote or disrupt protein-protein interactions (44, 45, 46).

Ser-916 at the C-terminal end was found to be autophosphorylated in vitro and in vivo both in GST-PKD and in GST-catPKD (Fig 1 and Table 2). Interestingly, Ser-916 is not in a carboxy-terminal phosphorylation site consensus sequence FXXF(S/T)(Y/F) identified in other kinases (PKB, cPKCs, nPKCs and p70\(^{S6K}\)) (47). Using an anti-phospho-Ser-916 antibody, it was shown that phosphorylation of Ser-916 correlated with PKD activity and was induced by phorbol ester or by antigen receptor triggering in lymphocytes (24). Replacement of Ser-916 by alanine or glutamate demonstrated that autophosphorylation of Ser-916 is not required for activity or for in vivo activation by phorbol ester (Table 1). Likewise in PKC\(\beta\) II, where two autophosphorylation sites are located at the carboxyl terminus, one site (Ser-660) does not control the kinase activity but rather plays a structural role, in both the active site and the regulatory region, by increasing the affinity for substrates, phosphatidylinerine and Ca\(^{2+}\) (9, 33). The S916A mutant showed a reduced sensitivity to proteolysis (Fig. 2) and to dephosphorylation by alkaline phosphatase (Fig 3), which indicates that the Ser-916 to alanine mutant might have a more closed conformation, and that a C-terminal negative charge would favor a more open structure. Moreover, the Ser-916A mutant exhibited a delayed time-dependent downregulation of its activity after phorbol ester stimulation (Fig. 4). We also showed that the downregulation of PKD activity is a reversible process, probably under the control of a protein phosphatase (Fig. 4). This is in agreement with our previous report showing that the activation of PKD can be fully reversed in vitro by protein phosphatases.
PP1c and PP2A c (15). There exist several examples of C-terminal kinase phosphorylation /dephosphorylation as a regulatory mechanism for kinase activity downregulation. For PKBα, downregulation of the kinase activity occurs via dephosphorylation of the two major regulatory phosphorylation sites (Thr-308 in the activation loop and Ser-473 at the C-terminal end). It is known that their mutation to aspartate leads to a constitutively active enzyme that cannot be downregulated (48). As a variation on this theme, IKKβ also contains C-terminal autophosphorylation sites involved in the downregulation of its kinase activity (49). However, replacement of these serines with alanine in IKKβ results in a mutant that remains active four times longer than the wild-type enzyme. Likewise, mutation of S916 to alanine in PKD instigates a slower downregulation of the kinase activity, while a negative charge at this position induced by phosphorylation or mutagenesis seems to favor the process (Fig. 4), possibly by inducing a conformational change. This could render other phosphorylation sites more accessible to protein phosphatases which are involved in the reversal of kinase activation. It should be mentioned that the downregulation of PKD activity was postponed but not abolished in the S916A mutant, indicating that additional mechanisms are involved.

14-3-3τ proteins have been proposed to associate with PKCµ and to negatively regulate PKCµ kinase activity (50). Mutational analysis suggested that this association involved two serine pairs (serines 205/208 and 219/223 in PKCµ) and both these pairs of serine residues were proposed to be autophosphorylation sites of PKCµ. However, in these studies, only combinations of double mutants were tested, which cannot pin-point the individual residues required for interaction with 14-3-3τ. Moreover the phosphorylated serine residue(s) were not positively identified. These two serine pairs are conserved in PKD and correspond to serines 203/206 and 217/221. Here we identified Ser-203 as an in vitro and in vivo autophosphorylation site in PKD. Ser-206 was detected as being non-phosphorylated (Table 2). We have no evidence that a serine residue in the second pair is phosphorylated. Moreover, we could identify and sequence peptides containing Ser-217 and Ser-221 in their non-phosphorylated states in autophosphorylated PKD.

We identified Ser-255 as a PDBu-induced transphosphorylation site in 32P-labeled cells, downstream of a PKC-dependent pathway (Table 2). Although the S255E mutant is not constitutively active, its activation by phorbol ester no longer requires PKC activity (Fig. 6).
The demonstration that of all the mutants tested, only the S255E mutant can still be activated by PDBu in the presence of PKC inhibitors, indicates that the stimulation of PKD by phorbol ester also encompasses events other than PKC-mediated phosphorylation. In view of the fact that PKD can be partially stimulated \textit{in vitro} by the addition of PS/PDBu micelles, one could envisage that PDBu uses a bifurcating pathway for the activation of PKD \textit{in vivo}. One signaling path leads to a PKC-dependent transphosphorylation of Ser-255, whereas the second path involves other PDBu dependent events perhaps involving direct PDBu binding to the PKD Zn fingers. Replacement of Ser-255 by Ala demonstrated that Ser-255 phosphorylation is not strictly required for PKD activation by PDBu (Table 1), but may be required for more efficient activation by the PKC pathway. This suggests that the activation of the S255A mutant may be the result of a phosphorylation of a neighbouring serine, and moreover points to the possibility that the PKC-dependent path of PKD-activation encompasses several equivalent phosphorylation sites in the vicinity of Ser-255. Multisite phosphorylation is a characteristic of many protein kinases e.g. in MAPKAP kinase-2 where any two of three sites must be phosphorylated to achieve maximal activation (51). A negative charge on the Ser-255 site (induced by mutagenesis or by phosphorylation) may be a prerequisite for the \textit{in vivo} activation of PKD via the second phorbol ester mediated pathway.

Our results demonstrate that Ser-744 and Ser-748 are not phosphorylated in full-length GST-PKD in response to phorbol ester stimulation (Table 2). However, these two sites are autophosphorylated \textit{in vivo} in GST-catPKD, which probably explains why GST-catPKD is highly active and cannot be further stimulated by the PDBu/PKC pathway (Table 1). PKD does not belong to the family of RD kinases, which are defined as kinases where the conserved catalytic aspartate is preceded by an arginine residue. Most RD kinases are regulated by phosphorylation in the activation loop (8). In the 3-D structures of several RD kinases, the arginine residue in the “RD motif” interacts with the phospho-amino-acid in the activation loop (e.g. cAPK, MAPK, CDK2) or with a corresponding acidic residue (e.g. PhK) to promote the correct positioning of the catalytic site residues (7). Non-RD kinases, like PKD, are proposed not to be regulated by phosphorylation in the activation loop (8). There is an interesting double substitution in twitchin, the only non-RD kinase of known structure, with the ion-pair seen in RD kinases being replaced by two uncharged residues (valine and
leucine) (52). This probably explains why this kinase is not regulated by phosphorylation in the activation loop. In PKD, an intermediate situation is found where the arginine of the RD motif is replaced by a cysteine, while Ser-748, a possible phosphorylation target corresponding to the phosphorylated residue in RD kinases, is present in the activation loop. If phosphorylation occurs on this serine in PKD, interaction with the cysteine residue would be weak. It is known from crystallographic data, that the activation loop plays an important role in substrate recognition and in the correct positioning of catalytic residues (7). Additional interactions mediated by phosphorylated Ser-744 and Ser-748 might promote structural changes in the catalytic site of GST-catPKD and induce a higher catalytic activity. Neither of these two sites lies in the highly conserved activation loop phosphorylation site consensus sequence T(F/L)CGT identified amongst the AGC family of kinases (47). Nevertheless, they may be important for the in vivo activation of PKD/PKCµ by diacetyl-glycerol-independent pathways such as the Gβγ-mediated regulation of PKD in Golgi structure and function (17) or form the basis of the in vitro stimulation of PKD by dextran sulfate (23). Our results may be at variance with earlier studies (23), but emphasize the need for positive identification of phosphorylation sites rather than indirect evidence from site-directed mutagenesis studies.

One might envisage an alternative pathway for PKD activation where a protease driven mechanism would be involved. Interestingly, one study has shown that stable PKCµ transfectants exhibit a reduced sensitivity to tumor-necrosis-factor (TNF)-induced apoptosis (16). The authors reported that PKCµ is stimulated by TNF and promotes the activation of NF-κB dependent genes, counteracting apoptotic signals. More recently, it was shown that treatment of cells with various apoptosis-inducing agents caused a caspase-3 mediated proteolytic cleavage of PKCµ, between the regulatory and catalytic domain (53). The caspase-3 cleavage site in PKCµ was determined by site-directed mutagenesis, but this site is not conserved in PKD. In the present report we show that PKD can be activated in vitro by limited proteolysis (Fig. 7). This irreversible activation could be due to the removal of the inhibitory regulatory domain and/or to the unmasking of new phosphorylation sites in the kinase domain. Indeed, autophosphorylation on Ser-744 and Ser-748, observed only with the isolated catalytic domain, could perhaps play a role in the activation of PKD by proteolysis which has the potential to generate free catalytic domain.
In conclusion, phosphorylation of PKD at particular sites may be an intricate mechanism for the selective control of its biological functions. More work is in progress on the hierarchy of the observed phosphorylations and their potential role for the association of PKD with other proteins. Finally, further studies will be needed to investigate whether other activating signalling pathways such as Gβγ subunits or caspase-mediated proteolysis lead to differential phosphorylation of PKD.

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FOOTNOTES

1 transphosphorylation refers to a phosphorylation catalyzed by another kinase.

2 The abbreviations used are: cAPK, cyclic AMP dependent protein kinase; IRK, insulin receptor kinase; PKB, protein kinase B; MAPK, mitogen activated protein kinase; PDK-1, 3-phosphoinositide-dependent protein kinase; TCA, trichloroacetic acid; MLCK, myosin light chain kinase; PDBu, phorbol 12,13-dibutyrate; PS, phosphatidyl-L-serine; PhK, phosphorylase kinase; CDK2, cyclin-dependent kinase 2; PAK, p21-activated protein kinase; PRK1, protein kinase C-related kinase-1; MEKK-1, mitogen-activated protein kinase kinase-1; p70^{S6K}, 70 kDa S6 kinase; cPKCs, conventional protein kinases C; nPKCs, novel protein kinases C; AEBSF, 4-(2-aminoethyl)-benzenesulfonfyl fluoride hydrochloride; BES, (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid); IKKB, IkB kinase.
FIGURE LEGENDS

Figure 1

Identification of Ser-916 as an in vitro phosphorylation site

The major $^3$P-labeled peak from the chymotryptic digestion of in vitro autophosphorylated GST-PKD was analysed by nanospray ESI-MS/MS and found to contain one phosphopeptide ion (P1 in table 2). (a) MS$^2$ spectrum of this mono charged phosphopeptide ion (m/z 883.4). A loss of 98 Da is observed (H$_3$PO$_4$) to produce an ion with m/z 785.3. (b) MS$^3$ spectrum of the ion arising from loss of 98 Da {m/z 785.3 of the mono charged ion in (a)}. The b$_4$ and b$_5$ fragments have a mass difference of 69 Da corresponding to dehydroalanine, identifying the product of phosphoserine after losing 98 Da. The “B” label denotes dehydroalanine, the “b” and “y” labels refer to ions containing the N-terminal or C-terminal ends of the molecule, respectively.

Figure 2

Sensitivity to trypsin proteolysis of wild-type, S916A and S916E recombinant GST-PKD preparations

Purified PKD (5 µg) from PDBu stimulated cells was incubated for 15 min at 30°C in buffer containing 20 mM Tris-Cl pH 8.0, 0.3 mM CaCl$_2$, and the indicated concentrations of trypsin. Proteins were separated by SDS-PAGE in 10% acrylamide and stained with Coomassie Brilliant Blue. Intact GST-PKD and the Mr 42000 fragment generated from the wild-type are indicated by arrows.

Figure 3

Sensitivity of wild-type, S916A and S916E recombinant GST-PKD preparations to alkaline phosphatase treatment

Purified wild-type (O), S916A (■) or S916E (▲) (10 µg of each) from PDBu stimulated cells were autophosphorylated in vitro as described in the experimental procedures section. Proteins were incubated at 30°C in buffer containing 50 mM Tris-Cl pH 9.0, 20 mM MgCl$_2$ with 25 U/ml of shrimp alkaline phosphatase. Aliquots were taken at the indicated times and proteins
were separated by SDS-PAGE in 10% acrylamide. The extents of phosphorylation of the GST-PKD bands were measured after Coomassie Blue staining and phosphorimaging (Molecular Dynamics). The results are the means of two separate experiments. 100 % corresponds to 0.40, 0.27 and 0.25 mole of phosphate incorporated per mol of wild-type, S916A and S916E, respectively.

**Figure 4**

**Time-course of downregulation of PKD activity after phorbol ester stimulation.**

HEK 293T cells transiently expressing wild-type (O), S916A (■) or S916E (▲) untagged PKD were stimulated with 500 nM PDBu for 15 min. After extensive washing with PBS, cells were lysed (time 0) or incubated in DMEM without PDBu for the indicated times and then lysed. PKD activity was measured after immunoprecipitation with a polyclonal antibody as described (15). 100% of PKD activity corresponds to the initial activity measured right after PDBu stimulation (time 0). The results are the means ± S.E.M for three separate determinations. Inset, relative activity of PKD in HEK 293T cells transiently expressing wild-type PKD. Closed bar, after PDBu stimulation; open bar, 6 hours after PDBu stimulation; hatched bar; 6 hours after PDBu stimulation and restimulated 15 min with PDBu. The results are the means ± S.E.M for three separate determinations. *P < 0.05 vs. wild-type at same time.

**Figure 5**

**Identification of Ser-203 as an in vivo phosphorylation site**

Purified GST-PKD was digested with trypsin and the peptides were separated and analysed on-line by capillary HPLC-ESI-MS/MS. The LC/MS data were scanned for ions with m/z predicted for the unphosphorylated, monophosphorylated, and diphosphorylated peptides containing Ser-203. After identifying candidate ions from this initial analysis, the sample was run a second time and the ions were selected for on-line CID in MS² and MS³ mode. (a) MS full scan for a phosphopeptide ion of m/z 777.3 (P5 in table 2). (b) MS³ spectrum of the ion arising from loss of 98 Da {m/z 728.3 of the double charged ion in (a)}. The y₁₂ and y₁₁
fragments have a mass difference of 69 Da that corresponds to the mass of dehydroalanine identifying Ser-203 as phosphoserine. For other details of the labeling, see legend to Fig. 1.

Figure 6
Inhibition of PDBu-induced activation of PKD by bisindolylmaleimide I
HEK 293T cells transiently expressing wild-type and mutated GST-PKD were incubated in DMEM with (open bars) or without (closed bars) 4 µM bisindolylmaleimide I (Gö 6850) for 2 hours. Cells were then treated with 1µM PDBu for 10 min or directly lysed (control). Lysis, purification of GST-PKD and PKD activity measurements were performed as described in the experimental section. Results are expressed as fold-activation of PKD versus control conditions (no PDBu). The results are the mean ± S.E.M for three separate determinations.

* P< 0.05 vs. condition without Gö 6850.

Figure 7
PKD activation by limited proteolysis
(a) Purified GST-PKD (25 μg) was incubated with 0.02 U/ml trypsin in a buffer containing 20 mM Tris, 0.3 mM CaCl₂, pH 8.0 at 30 °C. Aliquots were taken at the indicated time and analyzed by SDS-PAGE in 10% acrylamide. The gel was stained with Coomassie Brilliant Blue. The three major proteolytic fragments are indicated by arrows (Mr of 90000, 42000 and 26000).

(b) GST-PKD purified from unstimulated or PDBu-stimulated HEK 293T cells was incubated with 0.02 U/ml of trypsin (filled symbols) in a buffer containing 20 mM Tris, 0.3 mM CaCl₂, pH 8.0, at 30°C. Aliquots were taken at the indicated time and PKD activity was measured as described in the experimental procedures section, except that 1 mM AEBSF was included in the assay buffer to inhibit any further proteolysis. The results are representative of three separate determinations.

Figure 8
Localization of phosphorylation sites in wild-type and catalytic domain PKD
Schematic outline of the structural domains of PKD containing the two cysteine-rich domains (CRD), the pleckstrin homology (PH) domain and the kinase core (CAT). Phosphorylation sites are indicated by an open circle (autophosphorylation) or a closed circle (transphosphorylation). Proteins that associate with PKD are listed under the region of PKD with which they have been proposed to interact (17, 22, 50, 54, 55).
Table 1

Kinetic properties of PKD in the recombinant GST-tagged wild-type and mutant preparations under basal or PDBu stimulated conditions

HEK-293T cells were transiently transfected with vectors expressing GST-PKD, GST-catPKD and the different mutants of GST-PKD. The cells were stimulated with PDBu and the recombinant proteins purified as described under “Experimental Procedures”. Purified PKD activity was measured at 30°C in buffer containing 15 mM Tris-Cl pH 8.0, 5 mM MgCl2, 1mg/ml bovine serum albumin, 500 µM [γ-32P]MgATP (100 cpm/pmole) and 500 µM syntide-2 peptide (1). For the syntide-2 and MgATP saturation curves, the concentration of substrates were varied up to 10 times the $K_m$. The concentration of the other substrate was 500 µM, except for the S744A mutant, where the syntide-2 concentration was 1 mM. The values are the means ± S.E.M for at least three determinations.

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**Table 2**

Sequences of PKD phosphopeptides determined by ESI-MS/MS

Purified GST-PKD preparations, after *in vitro* autophosphorylation or directly after purification (*in vivo*), were submitted to trypsin or chymotrypsin in-solution digestions as described under “Experimental Procedures”. *In vitro* or *in vivo* sites were analysed by nanospray ESI-MS/MS or by on-line capillary HPLC-ESI-MS/MS, respectively. Phosphopeptides were identified by loss of 98 Da under collision induced dissociation and the phosphorylated residue was further identified by fragmentation in MS³ mode.

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(b) $\text{MS}^3$ of $[\text{M+H}^+] = 785.3$
(a) MS full scan for [M+2H\(^+\)] = 777.3

(b) MS\(^3\) of [M+2H\(^+\)] = 728.3
Regulation of Protein Kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis

Didier Vertommen, Mark Rider, Youping Ni, Etienne Waelkens, Wilfried Merlevede, Jackie R Vandenheede and Johan Van Lint

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