Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8572–8576, August 1994
Biochemistry

Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain

(signal transduction/bacterial expression/phorbol ester receptor/kinase domain)

ANGELA M. VALVERDE, JAMES SINNETT-SMITH, JOHAN VAN LINT, AND ENRIQUE ROZENGURT*

Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Communicated by Leon A. Heppel, May 13, 1994 (received for review April 1, 1994)

ABSTRACT **A serine/threonine protein kinase that binds phorbol esters and diacylglycerol (named protein kinase D, PKD) has been identified. PKD contains membrane localization signals and a cysteine-rich repeat sequence homologous to that seen in the regulatory domain of protein kinase C (PKC). A bacterially expressed N-terminal domain of PKD exhibited high-affinity phorbol ester binding activity (K_d = 35 nM). The diacylglycerol analog 1-oleoyl-2-acetylglycerol inhibited phorbol ester binding in a dose-dependent manner. The catalytic domain of PKD contains all characteristic sequence motifs of serine protein kinases but shows only a low degree of sequence similarity to PKCs. The highest identity is with the catalytic domain of myosin light-chain kinase from *Dictyostelium* (41%). The bacterially expressed catalytic domain of PKD efficiently phosphorylated the exogenous peptide substrate syntide 2 in serine but did not catalyze significant phosphorylation of a variety of other substrates used by PKCs and other major second messenger regulated kinases. PKD may be an unusual component in the transduction of diacylglycerol and phorbol ester signals.**

A rapid increase in the synthesis of lipid-derived second messengers is an important mechanism for transducing extracellular signals across the plasma membrane (1–3). The phospholipase C-mediated hydrolysis of inositol phospholipids is known to produce two second messengers: inositol 1,4,5-trisphosphate, which induces mobilization of calcium from intracellular stores (3), and diacylglycerol (DAG), which activates protein kinase C (PKC)—originally described as a Ca^{2+}-activated, phospholipid-dependent protein kinase (4). The early findings that the potent tumor promoters of the phorbol ester family can substitute for DAG in PKC activation and that the phorbol ester receptor and PKC copurify supported the hypothesis that the cellular target of the phorbol esters is PKC (4).

Subsequent studies revealed the diversity of the individual components of the DAG–PKC signal transduction pathway. PLC exists in several molecular forms that are regulated by guanine nucleotide binding proteins and tyrosine phosphorylation (5). Furthermore, DAG can be generated by alternative routes involving the hydrolysis of phosphatidylcholine (6). Molecular cloning has demonstrated the presence of multiple related PKC isoforms that are differentially expressed in cells and tissues (2, 7). All members of the PKC family—i.e., conventional PKCs (α, βI, βII, and γ), unusual PKCs (δ, ε, η, θ), and atypical PKCs (ζ, λ, ι)—possess a highly conserved catalytic domain. Most of the variation between the PKC subspecies occurs in the regulatory domain. The C1 region of this domain of both conventional and unconventional PKCs has a characteristic tandem repeat of zinc finger-like cysteine-rich motifs that is indispensable to confer phospholipid-dependent phorbol ester and DAG binding to these PKC isoforms (8–11). In contrast, atypical PKCs contain a single cysteine-rich motif, do not bind phorbol esters, and are not regulated by DAG (12–14). These studies emphasized the complexity of the signaling pathways initiated by DAG but did not exclude the possibility that other protein kinases, unrelated to the PKC family in their catalytic domain, could also play a role in mediation of the cellular effects of DAG and phorbol esters.

Here we report the molecular cloning, sequencing, and expression analysis of a serine/threonine protein kinase with unusual enzymological properties.† Our results identify a cellular target for the tumor-promoting phorbol esters and DAG (named protein kinase D, PKD) that may function in signal transduction of DAG-generating receptors.

MATERIALS AND METHODS

Cell Culture. Cultures of Swiss 3T3, 3T6, and tertiary whole mouse embryo cells were propagated and rendered quiescent as described (15).

PCR Cloning. PCR amplification was performed as described by Wilks (16) using a Swiss 3T3 fibroblast cDNA library cloned into the *Eco*RI site of λZAPII vector (17) as a template. The material in the 210-bp band was excised and purified and cloned into pBluescript (Stratagene). Plasmids containing PCR fragments were sequenced by the dideoxynucleotide chain-termination method (Sequenase version 2.0 kit) using adenosine 5′-[α-[^{35}S]thio]triphosphate. Sequences were searched with GenBank/EMBL data bases.

Library Screening. The PK17 PCR product was radiolabeled by random priming (10^8 cpm/μg) and used to screen 10^6 plaques from a λZAPII Swiss 3T3 fibroblast cDNA library by standard procedures (18). The clone that gave the strongest hybridization signal, SW-4, was radiolabeled by random priming and used to screen a λgt11 random-primed 5′ stretch cDNA mouse lung library (Clontech). This resulted in isolation of clone L6 (2.2 kb), which extended the sequence toward the 5′ end. Additional overlapping clones were found by successive rounds of screening of the same library with 5′ 300-bp PCR fragments.

Expression Constructs and Generation of Fusion Proteins. A 1032-bp PCR fragment comprising the entire catalytic domain of PKD and a 624-bp PCR fragment containing the two cysteine-rich motifs within the regulatory domain were inserted in-frame into the inducible bacterial expression vector

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; PDB, phorbol 12,13-dibutyrate; MLCK, myosin light-chain kinase; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; OAG, 1-oleoyl-2-acetylglycerol; PKD, protein kinase D.

*To whom all reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z34524).

pMAL-c2 (19). The maltose binding protein fusion proteins expressed in *Escherichia coli* were purified by affinity chromatography through an amylose resin column (New England Biolabs).

Materials. All restriction enzymes, ultrapure cesium chloride, guanidine isothiocyanate, and the Sequenase version 2.0 kit were from United States Biochemical. [^{3}H]Phorbol 12,13-dibutyrate ([^{3}H]PDB) (18.6 Ci/mmol; 1 Ci = 37 GBq) was obtained from Dupont. Oligonucleotide primers were synthesized by Ian Goldsmith at the Imperial Cancer Research Fund. Bacterial strains of *E. coli* (SURE; XL1-blue) and helper phage R408 were from Stratagene. Other items were from standard suppliers or as listed in the text.

RESULTS AND DISCUSSION

Identification of a Member of the Protein Kinase Family. Using a PCR cloning strategy originally designed to isolate different members of the tyrosine kinase family (16), we identified a product, PK17, that was not represented in the data bases searched and consequently it was used to screen a Swiss 3T3 cell library. Surprisingly, the clone that gave the strongest hybridization signal, SW-4, encoded the catalytic domain and a 3′ untranslated region of a putative serine/threonine kinase rather than a tyrosine kinase (see below). SW-4 cDNA was used as a probe to isolate additional clones from a mouse lung cDNA library that overlapped with the 5′ end of SW-4 (Fig. 1*A*).

The composite sequence comprising 3179 bp total and the deduced amino acid sequence are shown in Fig. 1*B*. A single transcript of 3.3 kb was detected in Northern blots of RNA from mouse lung (Fig. 2). This indicates that the composite sequence shown in Fig. 1 is nearly full length.

The 5′ end of the assembled sequence was extremely G+C-rich. Kozak (20) described such G+C-rich sequences in the 5′ region of the mRNAs encoding regulatory proteins. An ATG positioned within the highly G+C-rich region is a likely initiation codon, since it is surrounded by a good consensus sequence for translation initiation (20). Initiation at this position would result in an open reading frame of 2754 bases. This coding region specifies a protein of 918 amino acids with a predicted molecular mass of 102 kDa. A 42-bp stretch containing four in-frame stop codons is present at the 3′ end of the coding region. The first stop codon (TGA), at position 2880, is separated from the poly(A) tail by 281 bp of 3′ untranslated sequence.

Expression of PKD in Mouse Tissues and Cultured Cells. Lung and brain show a prominent expression of the PKD mRNA. Testis, kidney, heart, and skeletal muscle display intermediate levels of expression, whereas liver and spleen appear to contain very low levels of mRNA (Fig. 2 *Left*). A single transcript was also detected in tertiary cultures of

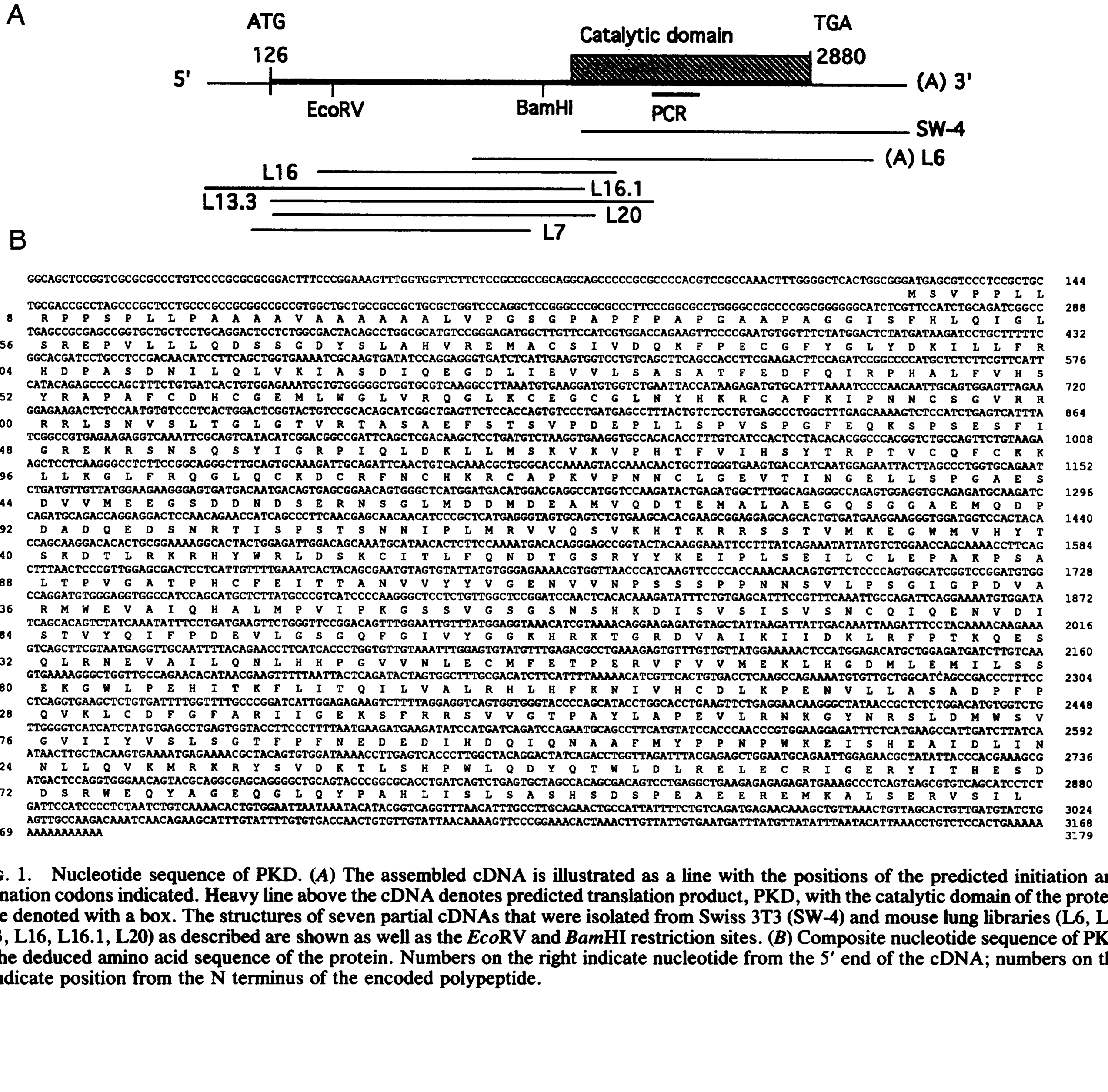

FIG. 1. Nucleotide sequence of PKD. (*A*) The assembled cDNA is illustrated as a line with the positions of the predicted initiation and termination codons indicated. Heavy line above the cDNA denotes predicted translation product, PKD, with the catalytic domain of the protein kinase denoted with a box. The structures of seven partial cDNAs that were isolated from Swiss 3T3 (SW-4) and mouse lung libraries (L6, L7, L13.3, L16, L16.1, L20) as described are shown as well as the *Eco*RV and *Bam*HI restriction sites. (*B*) Composite nucleotide sequence of PKD and the deduced amino acid sequence of the protein. Numbers on the right indicate nucleotide from the 5′ end of the cDNA; numbers on the left indicate position from the N terminus of the encoded polypeptide.

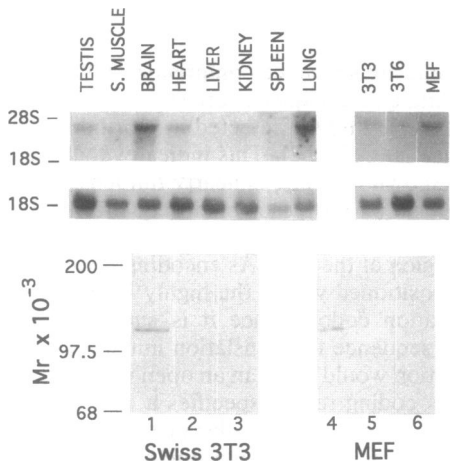

FIG. 2. Tissue and cell-type specificity of PKD expression. (*Upper*) Total RNA (20 μg) isolated (18) from several BALB/c mouse tissues (*Left*) and from quiescent Swiss 3T3 fibroblasts, 3T6 fibroblasts, and tertiary passage mouse embryo fibroblasts (MEF) (*Right*) was electrophoresed on a 1% agarose/6% formaldehyde gel and transferred onto Hybond-N$^+$ membranes. The hybridization probe was a gel-purified 1314-bp *Eco*RV/*Bam*HI cDNA fragment spanning the 5′ end of clone L16 radiolabeled to a specific activity of 3×10^8 cpm/μg by random priming. To determine the loading efficiency, the membranes were stripped and rehybridized with an 18S rRNA probe. S. muscle, smooth muscle. (*Lower*) PKD protein expression in Swiss 3T3 cells and mouse embryo fibroblasts. Cultures of Swiss 3T3 cells (6×10^6) or tertiary mouse embryo fibroblasts (MEF) (5×10^6 cells) were washed twice with phosphate-buffered saline at 4°C and lysed. PKD was immunoprecipitated from the clarified lysates with the PA-1 antibody in either the absence (lanes 1, 3, 4, and 6) or presence (lanes 2 and 5) of immunizing peptide (2 μg per μl of antiserum). Immunoprecipitates were analyzed by Western blotting using the PA-1 antiserum in either the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of immunizing peptide.

mouse embryo fibroblasts as well as in the mouse Swiss 3T3 and 3T6 cell lines (Fig. 2 *Right*).

To examine PKD protein expression in Swiss 3T3 cells and mouse embryo fibroblasts, lysates of these cells were subjected to immunoprecipitation with a polyclonal antiserum (PA-1) raised against the synthetic peptide EEREMKALSERVSIL that corresponds to the C-terminal region of the predicted amino acid sequence of PKD. The immunoprecipitates were then analyzed by immunoblotting using the same antiserum. PA-1 specifically recognized a single band migrating with an apparent molecular mass of 110 kDa, in agreement with the predicted molecular mass of PKD (Fig. 2). A fraction of the immunoreactive 110-kDa band was associated with cellular membranes (results not shown).

Structural Analysis. The putative catalytic domain of PKD contains a protein kinase consensus region between residues 589 and 845, which is composed of distinct subdomains (21). Searching the protein data bases with this sequence revealed the highest identity with the catalytic domain of myosin light-chain kinase (MLCK) of *Dictyostelium* (41%) (Fig. 3*A*). The rank order of homologies within the catalytic domain was as follows: MLCK (*Dictyostelium*) > Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) type II > CaM kinase type IV > cAMP-dependent protein kinase > phosphorylase B kinase. In particular, the catalytic domain of PKD exhibits only a low degree of similarity to the highly conserved regions characteristic of all members of the PKC family.

The salient feature of the deduced amino acid sequence of the N-terminal portion of PKD is a cysteine-rich repeat sequence ($HX_{12}CX_2CX_{13}CX_2CX_4HX_2CX_7C$) that exhibits homology with the cysteine-rich sequence tandemly repeated in the regulatory domain of all phorbol ester-sensitive PKCs (Fig. 3*B*). The length of the sequence separating the cysteine-rich domains in PKD (95 residues) is substantially longer than that of conventional PKCs (28 amino acids) or unconventional PKCs (35–36 amino acids). Other proteins that also contain a cysteine-rich, zinc finger-like motif with sequence identity to PKCs include the p21^{rac} GTPase-activating protein chimerin, the DAG kinase, the *Caenorhabditis elegans*

A

```
PKD    IFPDEVLGSGQFGIVCGGKHRKTGRDVAIKIIDKLRFPTKQESQLRNEVAILQNLHHPGVVNLECMFETPERVFVVMEKLHGDMLEMILS 678
MLCK   YEFKEELGRGAFSIVYLGENKQTKQRYAIKVINKSELGKDYEKNLKMEVDILKKVNHPNIIALKELFDTPEKLYLVMELVTGGELFDKIV  97

PKD    SEKGWLPEHITKFLITQILVALRHLHFKNIVHC DLKPENVLLASADPFPQVKLC DFGFARIIGEKSFRRSVVGTPAYL APEVLRNKGYNR 768
MLCK   -EKGSYSEADAANLVKKIVSAVGYLHGLNIVHR DLKPENLLLKSKENHLEVAIA DFGLSKIIGQTLVMQTACGTPSYV APEVLNATGYDK 186

PKD    SLDMWSVGVIIYVSLSGTFPFNEDEDIHD--QIQNAAFMYPPNPWKEISHEAIDLINNLLQVKMRK RYSVDKTLSHPWL    845
MLCK   EVDMWSIGVITYILLCGFPPFYGDTVPEIFEQIMEVNYEFPEEYWGGISKEAKDFIGKLLVVDVSK RLNATNALNHPWL    265
```

B

```
PKD                 PHALFVHSYRAPAFCDHCGEMLWG-LVRQGLKCEGCGLNYHKRCAFKIPNNC  194

mouse PKC alpha     DHKFIARPFKQPTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTFSC   86
rat PKC beta-1      NHKFTARPFKQPTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTSFC   86
rat PKC gamma       SHKFTARPFKQPTFCSHCTDFIWG-IGKQGLQCQVCSFVVHRRCHEFVTFEC   85
mouse PKC delta     NHEFIATPFGQPTFCSVCKEFVWG-LNKQGYKCRQCNAAIHKKCIDKIIGRC  208
mouse PKC epsilon   GHKFMATYLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRCHELIITKC  220
mouse PKC eta       GHKFMATYLRQPTYCSHCREFIWGVFGKQGYQCQVCTCVVHKRCHHLIVTAC  222
mouse PKC theta     CHEFTATFFPQPTFCSVCHEFVWG-LNKQGYQCRQCNAAIHKKCIDKVIAKC  209
mouse PKC zeta      GHLFQAKRFNRGAYCGQCSERIWG-LSRQGYRCINCKLLVHKRCHVLVPLTC  180

PKD                 PHTFVIHSYTRPTVCQFCKKLLKG-LFRQGLQCKDCRFNCHKRCAPKVPNNC  326

mouse PKC alfa      KHKFKIHTYGSPTFCDHCGSLLYG-LIHQGMKCDTCDMNVHKQCVINDPSLC  151
rat PKC beta I      KHKFKIHTYSSPTFCDHCGSLLYG-LIHQGMKCDTCMMNVHKRCVMNVPSLC  151
rat PKC gamma       KHKFRLHSYSSPTFCDHCGSLLYG-LVHQGMKCSCCEMNVHRRCVRSVPSLC  150
mouse PKC delta     PHRFKVYNYMSPTFCDHCGSLLWG-LVKQGLKCEDCGMNVHHKCREKVANLC  280
mouse PKC epsilon   PHKFGIHNYKVPTFCDHCGSLLWG-LLRQGLQCKVCKMNVHRRCETNVAPNC  292
mouse PKC eta       PHKFNVHNYKVPTFCDHCGSLLWG-IMRQGLQCKICKMNVHIRCQANVAPNC  295
mouse PKC theta     PHRFKVYNYKSPTFCEHCGTLLWG-LARQGLKCDACGMNVHHRCQTKVANLC  281

Consensus PKC       -H-F-------PTFC-HC---L/IWG----QG--C--C---VH-RC---V/I---C
```

FIG. 3. Alignment of PKD with related serine-threonine protein kinases. (*A*) Alignment of the catalytic domain of PKD and *Dictyostelium* MLCK. (*B*) Cysteine-rich domains of PKD were aligned with the cysteine-rich domains of PKC isoforms. Invariant residues forming a consensus sequence are also shown.

UNC13 gene product, and the oncogene products RAF and VAV (7, 10, 22).

In all PKCs, a pseudosubstrate motif characterized by an alanine flanked by basic amino acids is located 15 amino acids upstream from the beginning of the cysteine-rich domain. PKD does not contain sequences with homology to a typical PKC pseudosubstrate motif upstream of the cysteine-rich region, further illustrating the divergence of PKD from the PKC family. In contrast, the N-terminal domain of PKD contains hydrophobic regions that represent a putative leader sequence following the initiator methionine and a transmembrane domain.

We conclude that PKD is an unusual serine/threonine kinase with a striking structure. The catalytic domain is distantly related to Ca^{2+}-regulated protein kinases and clearly distinct from PKC. A second domain contains cysteine-rich motifs with a high degree of homology to the corresponding motifs of the C1 regulatory region of PKC.

Binding of [^{3}H]PDB to a Fusion Protein Containing the Cysteine-rich Domain. Next, we examined directly whether the N-terminal portion of PKD can function as a phorbol ester and DAG receptor. A fusion protein containing the cysteine-rich domain of PKD bound [^{3}H]PDB in a specific manner. Binding was directly proportional to the amount of fusion protein added to the assay mixture (Fig. 4*A*) and dependent on the presence of phosphatidylserine in the incubation mixture, but it was not affected by Ca^{2+}. The binding of [^{3}H]PDB to the fusion protein was displaced by unlabeled PDB in a dose-dependent fashion (Fig. 4*B*). In contrast, neither phorbol nor the biologically inactive phorbol 12-myristate 13-acetate 4-*O*-methyl ether competed with [^{3}H]PDB for binding to the fusion protein (Fig. 4*C*). As shown in Fig. 4*C*, the cell-permeant DAG analogue 1-oleoyl-2-acetyl glycerol (OAG) inhibited [^{3}H]PDB binding at concentrations (1–100 μg/ml) that elicit multiple responses,

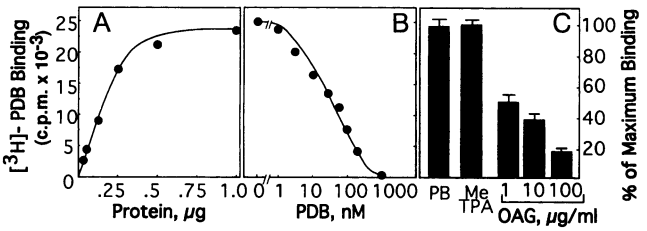

FIG. 4. Binding of [^{3}H]PDB to the PKD cysteine-rich domain fusion protein. (*A*) Binding of [^{3}H]PDB as a function of the amount of fusion protein containing the cysteine-rich domain. Binding to the fusion protein (25 ng to 1 μg as indicated) was determined by using [^{3}H]PDB at 10 nM in the presence of 20 mM Tris·HCl (pH 7.5) with phosphatidylserine (125 μg/ml) in a final vol of 200 μl. Incubations were carried out at 4°C for 30 min and then for a further 15 min at 4°C in the presence of 300 μl of DE52 equilibrated with 10 mM Tris·HCl (pH 7.5) (50:50 suspension). Incubations were terminated by rapid filtration on a Whatman GF/F filter disc followed by rapid washing with 20 mM Tris·HCl (pH 7.5) at 4°C. Similar results were obtained when bound [^{3}H]PDB was separated from free ligand using amylose resin instead of DE52. Radioactivity was determined by placing the filters in 10 ml of scintillation liquid. Nonspecific binding was estimated by addition of 10 μM unlabeled PDB to the incubation mixture. Phosphatidylserine was sonicated for 1 min at 4°C to form phospholipid vesicles. (*B*) Effect of unlabeled PDB on specific [^{3}H]PDB binding. The fusion protein containing the cysteine-rich domain (100 ng) was incubated with 10 nM [^{3}H]PDB in the presence of increasing amounts of unlabeled PDB as described above. Specific binding of [^{3}H]PDB was expressed as percentage of binding in the absence of unlabeled PDB. (*C*) Effect of 100 nM phorbol (PB), 100 nM phorbol 12-myristate 13-acetate 4-*O*-methyl ether (MeTPA), or OAG at various concentrations on specific [^{3}H]PDB binding. Values are expressed as percentage of specific binding in the absence of unlabeled analogs. Results represent means ± SE of five experiments.

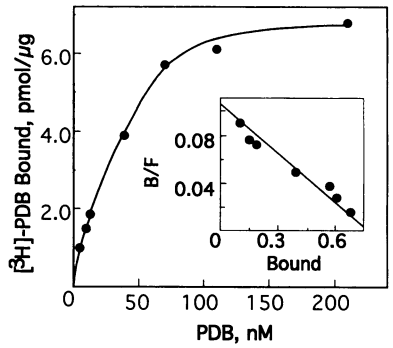

FIG. 5. Analysis of [^{3}H]PDB binding to the PKD cysteine-rich domain fusion protein. The fusion protein (100 ng) containing the cysteine-rich domain was incubated in the presence of various concentrations (3–200 nM) of [^{3}H]PDB for 30 min at 4°C in a final vol of 200 μl. Results presented are of a representative experiment, with each point determined in triplicate. Nonspecific binding was determined in the presence of 1000-fold excess unlabeled PDB and was ≈15% of the total binding. (*Inset*) Scatchard analysis of [^{3}H]PDB binding to the PKD cysteine-rich domain fusion protein. B/F, bound/free. Similar results were obtained in three independent experiments (K_d = 35 ± 7; mean ± SEM; n = 3). All other experimental details were as described in the legend to Fig. 4.

including mitogenesis, in cultured cells (23). Specific binding of [^{3}H]PDB to the fusion protein containing the cysteine-rich tandem repeat was saturable (Fig. 5). Scatchard analysis of the data revealed a K_d of 35 nM.

PKD Is a Protein Kinase. To verify directly that PKD possesses catalytic activity, we tested the ability of affinity-purified fusion protein containing the catalytic domain of PKD to phosphorylate a variety of exogenous substrates. As shown in Table 1, the catalytic domain of PKD efficiently phosphorylated syntide 2, a substrate (PLARTLSVAGLPGKK) also used by calmodulin-dependent protein kinases (22, 24, 25).

Sequence analysis of syntide 2 phosphorylated by the catalytic domain of PKD verified that phosphorylation occurred exclusively on serine (results not shown). The catalytic domain of PKD did not phosphorylate any other of the

Table 1. Relative phosphorylation of different substrates

Substrate	nmol·min^{-1}·mg^{-1}	% phosphorylation
Syntide 2	57	100
G peptide	0.7	1.2
APRTPGGRR	0.2	0.3
RRREEESEEE	0.1	0.2
GS-1 peptide	1.5	2.6
S6 peptide	0.7	1.2
PKC pseudosubstrate ε	0.1	0.2
Protamine	0.4	0.7
Myelin basic protein	0.8	1.4
Histone	0.5	0.9

PKD catalytic domain fusion protein (50 μg/ml) was incubated for 5 min with different protein kinase substrates as described in the legend to Fig. 6. All substrates were used at a final concentration of 2.5 mg/ml. G-peptide (SPQPSRRGSESSEE) corresponds to the primary sequence surrounding protein kinase A phosphorylation site 1 in the glycogen-binding subunit of the type 1 phosphatase. APRTPGGRR is specifically phosphorylated by the extracellular signal-regulated kinase. RRREEESEEE is specifically phosphorylated by casein kinase 2 (CK-2). GS-1 peptide (YRRAAVPPSPSLSRHSSPHQSEDEE) contains several phosphorylation sites for CK-2 and proline-directed kinases such as glycogen synthase kinase 3. S6 peptide (RRRLSSLR) corresponds to a C-terminal amino acid sequence of the eukaryotic ribosomal protein S6. Histones tested were histone IIAS, IIIS, and VIIS.

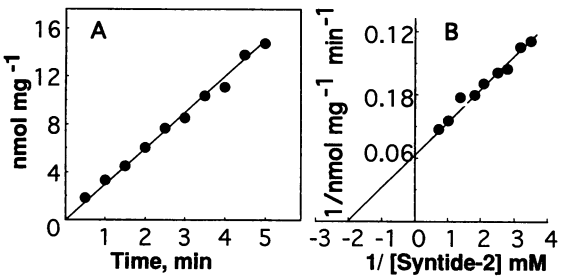

FIG. 6. Phosphorylation of syntide 2 by the catalytic domain of PKD: Time course and dependence on substrate concentration. (*A*) Time course. PKD catalytic domain fusion protein was incubated for various times with a phosphorylation mixture containing syntide 2. (*B*) Dependence on concentration. PKD catalytic domain fusion protein was incubated for 5 min with a phosphorylation mixture containing various concentrations of syntide 2. Kinase activity of the PKD catalytic domain fusion protein was measured by incubation at 30°C in a phosphorylation mixture containing 50 μg of PKD catalytic domain fusion protein per ml, 0.7 mM syntide 2, 125 μM [γ-^{32}P]ATP (specific activity, 300–600 cpm/pmol), and 15 mM $MgCl_2$. The kinase reaction was stopped by placing the reaction mixture on p81 phosphocellulose filter papers. [γ-^{32}P]ATP was separated from the labeled substrates by washing the papers in 75 mM H_3PO_4.

substrates tested (Table 1). These include a variety of substrates that are used by PKCs such as histone IIIS and a peptide based on the sequence of the pseudosubstrate region of PKCε. This pattern of substrate specificity clearly distinguishes PKD from the PKC family. In addition, PKD phosphorylated neither substrates containing a phosphorylation site for the cAMP-dependent protein kinase (G peptide and histone) nor a known substrate of the multifunctional CaM kinase II (myelin basic protein). Interestingly, PKD can also be distinguished from c-Raf1, which phosphorylates myelin basic protein more effectively than syntide 2 (26).

Affinity-purified fusion protein containing the catalytic domain of PKD phosphorylated syntide 2 in a time-dependent manner; linear kinetics were obtained up to 5 min of incubation (Fig. 6). A double reciprocal plot of the initial rate of syntide 2 phosphorylation as a function of syntide 2 concentration was linear, indicating that the catalytic domain of PKD obeys Michaelis–Menten kinetics. The apparent K_m for syntide 2 was 0.5 mM (Fig. 6).

Implications. Phorbol esters act as potent tumor promoters and induce a variety of responses in many cultured cell types including effects on ionic channels, second messenger production, cell–cell communication, membrane transport, protein phosphorylation, and cellular growth, morphology, differentiation, and transformation (2, 4, 27). The identification of another target for phorbol esters, PKD, whose mRNA is expressed in many organs and tissues as well as in cultured cells raises the possibility that some of the actions of phorbol esters could be mediated partially or exclusively by PKD.

One of the earliest responses of many cell types to extracellular stimuli is an increase in the synthesis of DAG, the physiological second messenger generated through multiple pathways (5, 6). PKD could provide an additional component in the flow of signal transmission and dissemination initiated by DAG.

Note. After this work was completed, we learned that a kinase with a high degree of identity to PKD has very recently been identified as a cDNA clone isolated from a human library and designated PKCμ (28). PKCμ has not been shown to bind phorbol esters. Consequently, further work will be required to determine whether these enzymes are functional homologs.

We thank Mr. M. Bouzyk for technical assistance. A.M.V. was a postdoctoral fellow of the Fundacion Ramon Areces and J.V.L. is a postdoctoral fellow of the European Molecular Biology Organization.

1. Majerus, P. W. (1992) *Annu. Rev. Biochem.* **61**, 225–250.
2. Nishizuka, Y. (1992) *Science* **258**, 607–614.
3. Berridge, M. J. (1993) *Nature (London)* **361**, 315–325.
4. Nishizuka, Y. (1989) *Cancer* **63**, 1892–1903.
5. Rhee, S. G. & Choi, K. D. (1992) *J. Biol. Chem.* **267**, 12393–12396.
6. Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4.
7. Hug, H. & Sarre, T. F. (1993) *Biochem. J.* **291**, 329–343.
8. Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4868–4871.
9. Burns, D. J. & Bell, R. M. (1991) *J. Biol. Chem.* **266**, 18330–18338.
10. Hubbard, S. R., Bishop, W. R., Kirschmeier, P., George, S. J., Cramer, S. P. & Hendrickson, W. A. (1991) *Science* **254**, 1776–1779.
11. Quest, A. F. G., Bardes, E. S. G. & Bell, R. M. (1994) *J. Biol. Chem.* **269**, 2953–2960.
12. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3099–3103.
13. Ways, D. K., Cook, P. P., Webster, C. & Parker, P. J. (1992) *J. Biol. Chem.* **267**, 4799–4805.
14. Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y. & Biden, T. J. (1993) *J. Biol. Chem.* **268**, 24296–24302.
15. Dicker, P. & Rozengurt, E. (1980) *Nature (London)* **287**, 607–612.
16. Wilks, A. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1603–1607.
17. Brooks, S. F., Herget, T., Erusalimsky, J. D. & Rozengurt, E. (1991) *EMBO J.* **10**, 2497–2505.
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
19. Guan, C., Li, P., Riggs, P. D. & Inouye, H. (1987) *Gene* **67**, 21–30.
20. Kozak, M. (1991) *J. Cell Biol.* **115**, 887–903.
21. Hanks, S. K. & Quinn, A. M. (1991) *Methods Enzymol.* **200**, 38–62.
22. Bruder, J. T., Heidecker, G. & Rapp, U. R. (1992) *Genes Dev.* **6**, 545–556.
23. Rozengurt, E., Rodriguez-Pena, A., Coombs, M. & Sinnett-Smith, J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5748–5752.
24. Mochizuki, H., Ito, T. & Hidaka, H. (1993) *J. Biol. Chem.* **268**, 9143–9147.
25. Lorca, T., Cruzalegui, F. H., Fesquet, D., Cavadore, J.-C., Méry, J., Means, A. & Dorée, M. (1993) *Nature (London)* **366**, 270–273.
26. Force, T., Bonventre, J. V., Heidecker, G., Rapp, U., Avruch, J. & Kyriakis, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1270–1274.
27. Rozengurt, E. (1986) *Science* **234**, 161–166.
28. Johannes, F.-J., Prestle, J., Eis, S., Oberhagemann, P. & Pfizenmaier, K. (1994) *J. Biol. Chem.* **269**, 6140–6148.