Protein Kinase D Induces Transcription through Direct Phosphorylation of the cAMP-response Element-binding Protein*

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Protein kinase D (PKD), a family of serine/threonine kinases, can be activated by a multitude of stimuli in a protein kinase C-dependent or -independent manner. PKD is involved in signal transduction pathways controlling cell proliferation, apoptosis, motility, and protein trafficking. Despite its versatile functions, few genuine in vivo substrates for PKD have been identified. In this study we demonstrate that the transcription factor cAMP-response element-binding protein (CREB) is a direct substrate for PKD. PKD1 and CREB interact in cells, and activated PKD1 provokes CREB phosphorylation at Ser-133 both in vitro and in vivo. A constitutive active mutant of PKD1 stimulates GAL4-CREB-mediated transcription in a Ser-133-dependent manner, activates CRE-responsive promoters, and increases the expression of CREB target genes. PKD1 also enhances transcription mediated by two other members of the CREB family, ATF-1 and CREM. Our results describe a novel mechanism for PKD-induced signaling through activation of the transcription factor CREB and suggest that stimulus-induced phosphorylation of CREB, reported to be mediated by protein kinase C, may involve downstream activated PKD.

The mammalian PKD† family of serine/threonine kinases includes the isoforms PKD1 (mouse PKD and human PKCμ), PKD2, and PKD3 (also named PKCν). PKD was originally considered to be a member of the PKC family (1, 2) but is now classified in the calcium/calmodulin-dependent kinase group based on sequence similarities in the kinase domain (3). The PKDs share a similar architecture consisting of a C-terminal catalytic domain, an N-terminal regulatory domain that encompasses two cysteine-rich regions (C1a and C1b), and a pleckstrin homology (PH) domain (4–7). A comparison of the amino acid sequences of PKD1–3 reveals that the highest homology lies in the catalytic domain, followed by C1a, C1b, and PH domain, suggesting that isoform-specific functions may be due to the regulatory part of the kinase (8).

PKD1 can be activated by several mechanisms. The most studied mechanism involves a sequential activation of phospholipase C that results in the generation of the second messengers inositol 1,4,5-triphosphate and diacylglycerol (DAG) and subsequent activation of the classical (α, βII, βII, and γ) and novel (δ, ε, η, and θ) PKC isoforms. Binding of DAG to the C1b domain of PKD1 directs its translocation to the plasma membrane where activated novel PKC phosphorylates PKD1 at Ser-744/748 in the activation loop, causing activation of the enzyme. The activated enzyme can be imported via its C1b motif into the nucleus, where it transiently accumulates before being exported to the cytosol through a CRM1-dependent nuclear export pathway that requires the PH domain of PKD (4–7). Mutations and deletions in the regulatory domain induce activation of PKD to various extents, and the entire regulatory domain has an inhibitory effect on the kinase activity (9). Indeed, PKD can also be activated through caspase-mediated cleavage of the regulatory domain (10). Gβγ subunits can activate PKD1 through direct interaction with the PH domain (11). However, a recent report suggested that the β1γ2-mediated activation of PKD required PKCη (12). Furthermore, PKD can be activated by bone morphogenetic protein 2 and endothelin-1 in a PKC-independent manner, but the mechanisms remain elusive (13, 14).

Depending on the cell type and external stimulus, PKD localizes to different cellular compartments (4). This suggests that PKD can regulate several cellular functions according to its cellular localization. PKD was shown to be involved in the fission of carriers from the trans-Golgi network to plasma membrane (15), and PKD-mediated phosphorylation of phosphatidylinositol 4-kinase IIIb results in enhanced vesicular stomatitis

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‡2 The abbreviations used are: PKD, protein kinase D; ca, constitutive active; CREB, cAMP-response element-binding protein; DAG, diacylglycerol; dn, dominant negative; PDGF, platelet-derived growth factor; PH, pleckstrin homology; RT, reverse transcriptase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; PKC, protein kinase C; GST, glutathione S-transferase; PH, pleckstrin homology, siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; PKA, protein kinase A; HDAC, histone deacetylase; PBS, phosphate-buffered saline; MBP, myelin basic protein; CREM, cAMP-response element modulator; CRE, cAMP-responsive element; APRT, adenine phosphoribosyltransferase.
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virus-G protein transport to the plasma membrane (16). PKD can also participate in cell motility (17, 18), and altered PKD1 activity in prostate cancer cells influences cellular aggregation (18). PKD mediates PDGF-induced suppression of epidermal growth factor–triggered c-Jun N-terminal kinase (JNK) activation in a cell-dependent manner (19–21). In addition, RIN1 phosphorylation by PKD1 has been demonstrated to result in activation of the Ras-MEK-ERK signaling pathway (22). Other studies have revealed a role for PKD in proliferation (23–25), osteoblastic cell differentiation (13), and apoptosis (4, 26). Finally, PKD may affect gene expression by inducing the nuclear exclusion of histone deacetylases (26–29).

Despite the diverse roles of PKD, relatively few in vivo substrates have been identified so far. These include the cytoplasmic proteins KidIns220 (kinase D interacting sub- strate of 220 kDa), VR1 (vanilloid receptor type I), troponin I, RIN1, phosphatidylinositol 4-kinase IIIβ, E-cadherin, HPK1 (hematopoietic progenitor kinase 1), and the nuclear substrates class II histone deacetylases HDAC5 and HDAC7 (16, 18, 22, 26, 27, 29–33).

CREB is a 43-kDa transcription factor whose activity is regulated by phosphorylation. Stimulus-induced phosphorylation of Ser-133, the major regulatory site, enables CREB to recruit CREB kinases (34, 35).

Using an oriented peptide library approach, Nishikawa et al. (36) determined the preferred substrate phosphorylation motif of PKD. This and similar studies revealed that PKD seems to preferentially phosphorylate substrates with a LXRXXX(T*/S*) consensus motif, where T* and S* denote the phosphoacceptor sites threonine or serine (36–38). Doppler et al. (38) used this knowledge and developed an antibody against a phosphopeptide with the PKD consensus motif. They identified Hsp27 as a novel substrate of PKD, but the antibody also detected an approximate 45-kDa protein in extracts from bombesin-1, bradykinin-1, PDGF-, phorbol ester-, or pervana- date-stimulated NIH3T3 cells (38). Interestingly, all these stimuli induce activation of PKD (39–42), as well as CREB Ser-133 phosphorylation (34), and the molecular mass of 45 kDa corresponds well with that of CREB (43 kDa). These observations prompted us to test whether CREB is a PKD substrate. Our results show that CREB is an in vitro and in vivo PKD substrate and that PKD activates CREB-dependent transcription in a Ser-133-dependent manner. The biological consequences of these findings are discussed.

EXPERIMENTAL PROCEDURES

Materials—12-O-Tetradecanoylphorbol-13-acetate (TPA), 4-β-phorbol 12,13-dibutyrate, platelet-derived growth factor (PDGF), myelin basic protein (MBP), sodium orthovanadate, and doxorubicin were purchased from Sigma. Fetal bovine serum and cell culture medium were obtained from Invitrogen. CDP-Star and MagicMark Western standard were from Applied Biosystems and Invitrogen, respectively. Antibodies against PKD1/PKCμ, phospho-PKD1/PKCμ (Ser-744/748), CREB, and phospho-CREB (Ser-133) were all obtained from Cell Signaling Technology, Inc. (Beverly, MA). Phospho-CREB (Ser-98)-specific antibodies were ordered from Sigma Genosys, which prepared affinity-purified antibodies from serum from rabbits immunized with keyhole limpet hemocyanin-conju- gated CKD/KRLF(pS)G. Luciferase assay kit was from PerkinElmer Life Sciences. G6976 and G6993 were from Calbiochem. Alkaline phosphatase-conjugated anti-rabbit antibody was purchased from DAKO (Denmark). Activated PKD1 and PKD2 were from Upstate (Medprose, Oslo, Norway), and the catalytic Ca subunit of PKA was purchased from Sigma.

Cells—COS-1 (ATCC CRL-1650), HEK293 (ATCC CRL-1573) and SK-N-DZ cells (ATCC CRL-2149) were all purchased from American Type Culture Collection Manassas, VA. The stably transfected A431-PKD1 cells have previously been described (43). All the cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and 100 μg/ml streptomycin (Invitrogen) in a CO2 incubator (5% CO2) at 37 °C. The stimulants pervanadate was freshly prepared for each experiment according to (44).

Purification of Activated PKD1—PKD1 was purified from 293T cells transfected with a GST-PKD1 expression vector (pGMEX-T3-PKD). Transfected cells were treated with 0.5 μM 4-β-phorbol 12,13-dibutyrate for 10 min before they were lysed. Cells were washed twice in PBS, followed by scraping them in buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 50 mM NaF, 5 mM sodium pyrophos- phate, 0.2 μM microcystin, 0.27 μM sucrose, 1 mM (2-amino-ethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, 15 μM β-mercaptoethanol, 1% Triton X-100). Extracts were cleared by centrifugation (20 min, 15,000 × g, 4 °C). Cleared lysate was incubated for 1.5–2 h with glutathione-Sepharose beads (Amersham Biosciences) on a rotating wheel at 4 °C. The beads were washed twice (each 20 min, rotation at 4 °C), first in buffer A with 0.5 μM NaCl and a second wash in buffer B (250 mM Tris, pH 8.5, 0.1 mM EGTA, 10% glycerol, 0.27 μM sucrose, 15 μM β-mercaptoethanol, 0.2 mM NaCl, 0.1% Triton X-100). GST- PKD1 was eluted from the beads with 1 bed volume of buffer B containing 40 mM reduced glutathione (Sigma) (30 min, rotation at 4 °C). The elution step was repeated once.

Plasmids—The plasmids GAL4-CREB, GAL4-CREB S133A, and pG5E1B Luc have been described previously (45). The empty expression vector pSR and the plasmid pSR Ca containing the catalytic Ca subunit from PKA, were the kind gifts from Dr. T. Jahnsen and Dr. K. Taske´n (46). The GST-CREB plasmid (amino acids 1–261 of CREB) was a kind gift from Dr. Michael Comb (47). The plasmids pcDNA3.1, pEGFP-C1, pCMV- CREB, pCMV-K-CREB, and the pGEX vector were purchased from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. pEGFP-N1 and pEGFP-C1 were the kind gifts from Dr. T. Jahnsen and Dr. R. Taskén (46). The GST-CREB plasmid (amino acids 1–261 of CREB) was a kind gift from Dr. Michael Comb (47). The plasmids pcDNA3.1, pEGFP-C1, pCMV-CREB, pCMV-K-CREB, and the pGEX vector were purchased from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively. EGFP-MK5L337 has been described previously (48) Constitutive active PKD (744/748 E/E mutant) and dominant negative PKD1 (K618N mutant) were cloned as XbaI/EcoRI fragments from Invitrogen, Clontech, and GE Healthcare, respectively.
VP16-CREB was created by subcloning of the ~1-kb EcoRI and BamHI fragment from CREB-pGADTP into VP16 (Clontech). CREB-pGADTP was prepared as follows; PCR was performed with GAL4-CREB as a template, using the primers 5'-ATGACATGGATTTGGCAGACAAAC-3' and 5'-CTTGGCCATAGGATCCTTAAAGT-3', which includes a restriction site for EcoRI and BamHI respectively. The resulting PCR product was digested with EcoRI and BamHI and sub-cloned into pGADT7 (Clontech). To clone the PH-KD domain of PKD1 (corresponding to the fragment generated by cleavage with caspase-3, consisting of amino acid residues 427–918 of mouse PKD1), PCR on full-length PKD cDNA was performed using the primers 5’-GCA CAC GAA GGC GTC GAC CAG CAC TGT G-3’ and 5’-CCA GGT CTG ATA GAG CTC TAG CCA AGG GTG ACT C-3’. The PCR product was digested with Sall and ScaI and cloned in the corresponding sites of pSL1180. The PH-KD sequences were then excised with EcoRI and XhoI and ligated into the corresponding sites of pcDNA3.1 to generate the eukaryotic expression vector for the PH-KD fragment of PKD1.

Site-directed Mutagenesis—All PCR-based site-directed mutageneses were performed using Stratagene QuikChange according to the instructions of the manufacturer (Stratagene, La Jolla, CA). The complementary primer sets for the generation of S89A, S98A, and S121A mutations in GAL4-CREB and GAL4-CREBS133A or GST-CREB expression plasmids were as follows: S89A, 5’-CAGTTCAGTCCGATGCAAGGACTTAAAAGAC-3’; S98A, 5’-GGACTTTAAAAAGACTTTCGCCGGCACTCAGATTCCACC-3’; S121A, 5’-GGAGTCTGTGATATGGTTACTGATGCGCAAACAGGG-3’ (only one strand is shown). All the mutations were verified by sequencing.

Sequencing—Cycle sequencing was performed using the Big Dye sequencing kit (PerkinElmer Life Sciences). Sequencing reactions were analyzed on an ABI377 Prism Sequencer (PerkinElmer Life Sciences).

In Vitro Kinase Assay—In vitro kinase assay was performed as outlined before (43). GST fusion proteins were purified from *Escherichia coli* BL21 extracts using glutathione-agarose beads according to the instructions of the manufacturer. Equal amounts of GST-CREB and GST-CREB mutants were used in all experiments. Distinct proteolytic bands were obtained, which is in agreement with others (50, 51).

Immunoprecipitation—Confluent 10-cm plates with HEK293 cells transfected with appropriate constructs were washed twice in ice-cold PBS and harvested in a cold lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and Complete protease inhibitor mixture (Roche Applied Science) (53). Lysates were clarified by centrifugation at 10,000 × g for 10 min. Lysates were precleared with random but irrelevant antibody for 1 h at 4 °C followed by precipitation of the nonspecific complexes with protein G-Sepharose (Amersham Biosciences). The precleared lysates were then incubated with anti-CREB protein for 1 h at 4 °C, followed by precipitation of the complexes with protein G-Sepharose. The immune complexes were washed three times in ice-cold lysis buffer (see above) and twice in ice-cold 50 mM Tris, pH 8.0, and subsequently subjected to immunoblot analysis.

Immunoblot Analysis—Immunoblot analyses were performed on immune complexes or extracts derived from cells grown in 6-well plates. The cells were washed twice in PBS, harvested in 80 μl of lysis buffer (0.25 mM dithiothreitol, 1:1 NuPAGE lithium dodecyl sulfate sample buffer (four times), and double distilled H2O), heated to 70 °C for 10 min, and sonicated for 4 s. Samples were analyzed by SDS-PAGE (4–12% NuPAGE; Invitrogen) and transferred to 0.45-μm pore size polyvinylidene difluoride membrane (Millipore). The blots were blocked with 5% nonfat dry milk (Nestlé), 0.1% Tween 20 (blocking buffer) for 1 h at room temperature and incubated with primary antibodies (1:1000) overnight at 4 °C in blocking buffer. The blots were subsequently incubated for 1 h in room temperature with alkaline phosphate-conjugated anti-rabbit antibodies (DAKO, Denmark) in blocking buffer before exposure to chemiluminescence substrate CDP-Star. To verify equal protein loading, the membranes were stripped for 5 min in 0.2 M NaOH, washed three times in PBS with 0.1% Tween 20 (PBST), blocked, and reprobed with anti-CREB antibody.

RNA Isolation and Reverse Transcriptase (RT)-PCR—Total RNA from transfected HEK293 cells was isolated using the Nucleospin RNA II purification kit (Clontech and Takara Bio Inc., Shiga, Japan) according to the manufacturers’ protocol. Two μg of RNA was reverse-transcribed using iScript cDNA synthesis kit (Biocompare, Bio-Rad) and subjected to PCR. The primers for APRT and the PCR conditions have been described previously (49). To amplify Nur77 cDNA, the following primers were used: 5’-TCTGCTCAGGCTGGTGCTAC-3’ (forward) and 5’-GGCACAAAGTCTCAGCTTGG-3’ (reverse). The PCR cycling conditions were 30 times at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR products were visualized on an ethidium bromide-stained agarose gel.

RESULTS

**PKD1 Phosphorylates CREB in Vitro at Two Residues**—PKD1 is a protein kinase that has unique substrate specificity, with a consensus sequence LXRXX(T/S*), where T*/S* denotes the target threonine and serine for phosphorylation, respectively (36–38). Interestingly, CREB Ser-133 is contained within the PKD consensus motif LSRRPS*, and a study with antibodies against phosphopeptide with PKD consensus motif detected a 45-kDa protein in extracts from bombesin-, bradykinin-, PDGF-, phorbol 12-myristate 13-acetate-, or pervanadate-stimulated NIH3T3 cells (38). All these stimuli induce activation of PKD as well as phosphorylation of CREB at Ser-133 (34, 39–42). This prompted us to test whether purified activated PKD1 or its isoform PKD2 could phosphorylate CREB in vitro. To ensure that purified PKD was active, myelin basic protein (MBP) was included in the experiment. The *in vitro* kinase
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FIGURE 1. PKD1 phosphorylates CREB at Ser-133 and Ser-98 in vitro. A, active PKD1 and PKD2 from Upstate were incubated with GST alone, GST-CREB fusion protein, or MBP in the presence of [γ-32P]ATP. The in vitro phosphorylated proteins were separated on SDS-PAGE and visualized by autoradiography. Lanes 1 and 2, MBP; lanes 3 and 4, GST; lanes 5 and 6, GST-CREB. B, purified activated PKD1 from phorbol ester-treated 293T cells and PKA were incubated with MBP, GST, or GST-CREB wild-type or mutants in the presence of [γ-32P]ATP. Phosphorylated proteins were separated on SDS-PAGE and visualized by autoradiography. Lane 1, MBP; lane 2, GST with activated PKD1; lane 3, GST-CREB with purified catalytic subunit of PKA; lane 4, GST-CREB with activated PKD1; lane 5, GST-CREBS133A mutant with activated PKD1; lane 6, GST-CREBS121A/S133A mutant with activated PKD1; lane 7, GST-CREBS98A/S133A with activated PKD1. C, purified active PKD1 from phorbol ester-treated 293T cells and GST-CREB proteins used in the kinase assay. Lane 1, GST-CREB and GST-CREBS133A but not GST-CREBS98A or GST-CREBS121A/S133A; lane 2, GST-CREBS121A/S133A, GST-CREBS98A, GST-CREBS98A/S133A, and GST-CREBS98A/S133A with activated PKD1; and lane 4, GST-CREBS98A/S133A with activated PKD1. D, GST-CREBS98A and GST-CREBS98A/S133A were incubated with either GST-CREB or GST-CREB mutants. Western blots were performed using phospho-Ser-133- (upper panel) or phospho-Ser-98 (lower panel)-specific antibodies. Lane 1, GST-CREB with activated PKD1; lane 2, GST-CREBS98A with activated PKD1; lane 3, GST-CREBS133A with activated PKD1; and lane 4, GST-CREBS98A/S133A with activated PKD1.

PKD-stimulating Agents Induce CREB Ser-133 Phosphorylation—Several growth factors like PDGF and regulatory peptides, as well as oxidative stress, have been shown to result in PKC-mediated activation of PKD (7). Pervanadate is a stim-
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Induced by all three stimuli in these cells (Fig. 2A, left panel). The same experiment was performed with TPA as a stimulus in SK-N-DZ cells (Fig. 2A, right panel). Also in these cells, stimulation with TPA resulted in both CREB and PKD1 phosphorylation, suggesting that the PKD-induced CREB phosphorylation is not cell-specific.

To further evaluate the contribution of PKD in stimuli-induced CREB phosphorylation, we assayed the phosphoserine 133-CREB levels in extracts of PDGF- or TPA-stimulated A431-PKD1 and TPA-stimulated SK-N-DZ cells in the absence or presence of the inhibitors G56975 (inhibits PKCα and -β and PKD) and G56976 (inhibits PKCα, β, γ, δ, or ζ). As can be seen in Fig. 2B, both inhibitors reduced the level of phosphorylated CREB in both PDGF- and TPA-stimulated cells, but not the total level of protein (Fig. 2B, upper versus lower panel). The presence of the inhibitors alone did not influence the phosphorylation of CREB neither in A431-PKD1 cells (Fig. 2B, left) nor SK-N-DZ cells (results not shown). The inhibition with G56976 reduced phosphorylation of CREB more than G56973, which may suggest the involvement of PKD in PDGF- or TPA-provoked CREB phosphorylation. PKCs (α, β, γ, δ, or ζ), although to a lesser extent than PKD, are also involved in PDGF- or TPA-induced CREB phosphorylation because the phospho-Ser-133 levels were diminished in the presence of G56976. The phosphospecific CREB antibodies cross-react with phosphorylated ATF-1. As can be seen in Fig. 2A,
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FIGURE 3. PKD1 interacts with and phosphorylates CREB at Ser-133 in vivo. A, HEK293 cells were transfected with pCMV-CREB (8 μg), and either pcDNA3 (8 μg), caPKD1 (8 μg), EGFP-empty vector (8 μg), or EGFP-MK5L337 (8 μg) expression vector. Whole cell extract was immunoprecipitated with anti-CREB antibodies and protein-Sepharose G beads. Resulting complexes were examined by SDS-PAGE and immunoblotted with anti-PKD1 antibodies. Left and right panel, lane 1, 5% of the input of the fraction with caPKD1-transfected cells (left panel) or EGFP-MK5L337-transfected cells (right panel); lane 2, co-immunoprecipitated (co-IP) PKD1 in caPKD1-transfected cells (left panel) or EGFP-MK5L337-transfected cells (right panel); lane 3, 5% input of the fraction with pcDNA3- (left panel) or EGFP-transfected cells (right panel); lane 4, co-immunoprecipitation of pcDNA3-transfected cells (left panel) or EGFP-transfected cells (right panel); B, A431-PKD1 cells (left panel) and COS-1 (right panel) were co-transfected with either empty vector (1 μg) (lane 1, both panels), the dnPKD (lane 2, both panels) (1 μg), or caPKD1 expression plasmid (1 μg) (lane 3, both panels) and the expression plasmid for chimeric GAL4-CREB or GAL4-CREBS133A (0.5 μg) (lanes 1–3 and 4–6, respectively). The fusion protein contains the DNA binding domain of GAL4 (residues 1–147) fused to full-length wild-type CREB or CREB in which serine-133 is replaced by alanine. Western blot was performed, and the level of phosphorylation was determined using a phosphoserine 133-specific antibody. To verify equal loading and blotting of the samples, the membrane was stripped and re-incubated with anti-CREB antibodies (middle panel). Finally, the expression of PKD was determined by PKD-specific antibodies (lower panel).

3-mediated proteolytic cleavage of PKD1 generates fragments consisting of the N-terminal region and the PH domain together with the kinase domain, the latter fragment possesses full catalytic activity (60). We wanted to explore whether doxorubicin could trigger PKD-mediated CREB phosphorylation. For this purpose, we assayed the phosphoserine 133-CREB levels in extracts of doxorubicin-stimulated A431-PKD1 cells in the absence or presence of Gø6976 or Gø6983. The results show that doxorubicin induces phosphorylation of CREB and ATF-1. Reduced levels of phosphorylated CREB and ATF-1 were observed in both Gø6976- and Gø6983-treated cells. Again, a stronger decrease in CREB and ATF-1 phosphorylation was observed in the presence of Gø6976 compared with Gø6983 (Fig. 2C). This indicates that PKD is involved in doxorubicin-induced CREB phosphorylation.

PKD1 could also phosphorylate CREB at Ser-98 in vitro (see Fig. 1). Phosphospecific antibodies raised against phospho-Ser-98 detected in vitro PKD1-phosphorylated CREB (Fig. 1D), but we were unable to detect phosphorylation of this site in cells treated with several PKD1 activating stimuli (results not shown) (see “Discussion”).

PKD1 Phosphorylates CREB at Ser-133 in Vivo—To corroborate the role of PKD1 as a CREB kinase, we examined direct interaction between PKD1 and CREB in vivo by co-immunoprecipitation experiments. HEK293 cells were transfected with pCMV-CREB and either empty vector pcDNA3.1 or an expression vector encoding a constitutive active (ca) PKD1 mutant, detected against endogenous CREB and exogenous PKD in COS-1 cells, and finally the interaction was confirmed by co-immunoprecipitation of endogenous PKD1 and CREB in A431-PKD1 cells (results not shown). We also performed the reciprocal experiment using anti-PKD antibodies to immunoprecipitate, but without success, which may be because of the antibody quality (result not shown).

To test whether activated PKD1 can also phosphorylate CREB in vitro at Ser-133, we co-transfected A431-PKD1 and COS-1 cells with an expression plasmid encoding caPKD1, dpPKD1, or pcDNA3.1 together with either an expression plasmid for GAL4-CREB or for GAL4-CREBS133A fusion protein. Western blot analysis with phospho-Ser-133-specific CREB antibody performed on extracts of transfected cells revealed that ectopic expression of activated PKD1 increased phosphorylation of GAL4-CREB at Ser-133 in both cell lines (Fig. 3B, lanes 3 in left and right panels). No increase in phosphorylation of GAL4-CREB was detected in the presence of empty vector or dpPKD (Fig. 3B, lanes 1 and 2, respectively, in left and right panels). To ensure that increased phospho-Ser-133 CREB levels in the presence of caPKD1 were not the result of unequal loading, the membranes were stripped and re-probed with antibodies against CREB (Fig. 3B, middle lane in left and right panels). The presence of endogenous or exogenous PKD1 was finally verified by antibodies against PKD1, which confirmed equal exogenous expression of both dpPKD and caPKD. We also included similar experiments where we used phospho-Ser-
98-specific antibody. However, the phospho-Ser-98-specific antibodies were unable to detect phosphorylation of this site in vivo in the presence of ectopically expressed activated PKD1. In conclusion, these experiments confirm the involvement of PKD1 in CREB phosphorylation of Ser-133 in vivo.

Activated PKD1 Enhances CREB-mediated Transcription—CREB is a substrate for a plethora of different kinases, and signal-induced phosphorylation of Ser-133 is necessary but not always sufficient to activate CREB-mediated transcription (reviewed in Refs. 34 and 61). This urged us to investigate whether phosphorylation of CREB by PKD1 increased the transcriptional potentials of CREB. Transient transfection studies performed in SK-N-DZ and A431-PKD1 cells revealed that co-expression of caPKD1, but not dnPKD1, stimulated GAL4-CREB-dependent transcription (Fig. 4A). Empty vector or an expression vector with wild-type PKD1 was unable to stimulate CREB-driven transcription (results not shown). PKD1-induced transcription activation was mediated by CREB and not by the
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basal transcriptional machinery or the GAL4 moiety because luciferase activities in the presence of the DNA-binding domain of GAL4 were comparable whether or not activated PKD1 was co-expressed (Fig. 4A, bars labeled pM). The induction of CREB-mediated transcription was Ser-133-dependent, because caPKD1 failed to stimulate GAL4-CREBS133A-driven transcription. Similar results were obtained with co-transfection studies in COS-1 cells (results not shown), indicating that PKD1-induced CREB activation is not cell-specific. To evaluate the amplitude of caPKD1-induced increase in CREB transcriptional potential, we included an experiment where we compared the transactivation potential of caPKD1 to the CREB subunit of protein kinase A (PKA), the first identified CRE kinase (35, 62). As shown in Fig. 4B, caPKD1 induced CREB transactivation potential to comparable levels as PKA. Ectopic expression of the PH kinase fragment of PKD1 also stimulated GAL4-CREB-mediated transcription (Fig. 4C).

CREB is a member of a family of proteins that also consists of CREM (cAMP-response element modulator) and ATF-1 (activating transcription factor). CREB and ATF-1 are expressed ubiquitously, whereas CREM has a more restricted expression pattern. All the members of the CREB family have similar functional domains and can be regulated by phosphorylation (61). Moreover, the phospho-Ser-133 antibodies that cross-react with the corresponding phospho-Ser-63 in ATF-1 also detected the phosphorylated form of ATF-1 in cells exposed to the PKD activators TPA and doxorubicin (Fig. 2). Therefore, we wanted to investigate whether PKD1 could transactivate the other CREB family members. HEK293 cells were transfected with dnPKD or caPKD and GAL4-CREB, GAL4-CREM, or GAL4-ATF-1. The transactivation activity of all the tested CREB family members was induced in the presence of caPKD, albeit with different amplitude, CREB being most responsive to caPKD1 (Fig. 4D).

From these experiments we conclude that constitutively activated PKD1, as well as the kinase-active PH-KD fragment, can transactivate GAL4-CREB in a Ser-133-dependent manner. Moreover, caPKD1 can enhance the transcriptional activities of GAL4-CREM and GAL4-ATF-1.

PKD1 Induces Expression of CRE-driven Promoter—The CREB family of transcription factors regulates promoters containing an 8-bp palindromic sequence known as the cAMP-responsive element (CRE) (61). We wanted to explore whether PKD1 could stimulate the activity of a CRE-driven promoter. SK-N-DZ cells were transfected with a reporter plasmid under the control of a cAMP-responsive promoter (pCRE-luc). The same reporter plasmid but with mutations in the CRE (pmutCRE-luc), which prevent CREB binding (49), was included as control for the specificity of caPKD1-induced transcription. As depicted in Fig. 5, co-expression of caPKD1 and CRE-luc resulted in a moderate (2.5-fold) but reproducible transactivation of the reporter. The transactivation was dependent on a functional CRE element, as pmutCREluc showed no significant increase in trans-activity by co-expression of caPKD1 compared with dnPKD1. Co-expression of the catalytic subunit of PKA (Ca) resulted in a 3.5-fold increase in luciferase activity compared with the activity measured in cells co-transfected with empty vector (pSR). These results suggest that PKD1 can induce a cAMP-responsive promoter by modulating the activity of CRE-binding proteins.

Activated PKD1 Induces Transcription of Nur77—Finally, we wanted to investigate whether PKD1 could stimulate transcription of cellular genes through direct phosphorylation of CREB. For this purpose, we ectopically expressed caPKD and monitored the expression of the CREB-responsive gene Nur77 (63). High transfection efficiency is favored in order to evaluate the effect of transient ectopic expression of PKD1 on transcription of endogenous genes. Therefore, we transfected the easily transfectable HEK293 cells with either caPKD, the dominant negative CREB mutants A-CREB or K-CREB, or combinations of these. A-CREB is a nonphosphorylatable mutant where Ser-133 is mutated into alanine, whereas K-CREB is a non-DNA binding CREB mutant that can dimerize with endogenous wild-type CREB and inhibit its binding to CRE (61, 64). TPA is well known PKD activator, and TPA stimulation results in CREB Ser-133 phosphorylation in several cells (34). Untreated or TPA-treated HEK293 cells were therefore included as controls. Total RNA was isolated, and a semi-quantitative RT-PCR was performed to determine the mRNA levels of Nur77. As shown in Fig. 6, TPA and the presence of caPKD1 clearly augmented Nur77 transcript levels. The PKD1-induced increase of Nur77 expression is mediated through CREB, as co-transfection with the dominant negative A-CREB or K-CREB resulted in reduced PKD1-induced Nur77 expression (Fig. 6). RT-PCR with APRT primers was included as a control and shows that the RNA quality and quantity was the same among the different samples. In accordance, caPKD1 also stimulated transcription of the ICER gene in a CREB-dependent manner (result not shown). These findings underscore that PKD1 can stimulate gene expression through direct activation of the transcription factor CREB.

DISCUSSION

PKD is a family of protein kinases involved in several processes, including protein transport, motility, apoptosis, prolif-
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HDAC5 phosphorylation in the PKD null cells (28). Similar functional redundancy may also explain that siRNA against PKD1 only resulted in 50% nuclear exclusion of HDAC5 in phenylephrine-treated NRMV cells (14). Thus, siRNA-mediated down-regulation of PKD1 in PDGF/TPA/pervanadate-treated cells will not influence the PKD2 and PKD3 levels, which can still mediate CREB Ser-133 phosphorylation. In conclusion, because of the lack of specific inhibitors/activator of PKD and possible PKD redundancy, we cannot exclude the contribution of other kinases in PDGF-, TPA-, pervanadate-, or doxorubicin-induced CREB phosphorylation.

In vivo CREB binding has been demonstrated for up to 4,000 different promoters of genes encoding proteins involved in transcription, metabolism, cell proliferation, differentiation, apoptosis, and the secretory pathway (75). Interestingly, PKD also contributes to these processes, implying that PKD may exert some of its function by inducing the expression of specific genes through modulating the activity of CREB. TPA-induced expression of Nur77 is mediated by PKD (26, 28, 29). Several studies have proven that activated PKD elicits nuclear exclusion of HDAC5 and HDAC7 (26, 27, 29), revealing a mechanism for the transactivation of Nur77 gene expression by PKD (26, 27, 29). Our study clearly reveals an additional mechanism for PKD-induced expression of Nur77, involving PKD-mediated phosphorylation of the transcription factor CREB. Indeed, the involvement of CREB in TPA-induced Nur77 expression is also seen in HeLa cells (73), and Nur77 is also up-regulated in PC12 cells in the presence of the constitutive active VP16-CREB (63). These findings support the notion that PKD can mediate translocation of phosphorylated HDACs and/or CREB (63). These findings support the notion that PKD can

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