The Pleckstrin Homology Domain of Protein Kinase D Interacts Preferentially with the η Isoform of Protein Kinase C*

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The results presented here demonstrate that protein kinase D (PKD) and PKCζ transiently coexpressed in COS-7 cells form complexes that can be immunoprecipitated from cell lysates using specific antisera to PKD or PKCζ. The presence of PKCζ in PKD immune complexes was initially detected by in vitro kinase assays which reveal the presence of an 80-kDa phosphorylated band in addition to the 110-kDa band corresponding to auto-phosphorylated PKD. The association between PKD and PKCζ was further verified by Western blot analysis and peptide phosphorylation assays that exploited the distinct substrate specificity between PKCs and PKD. By the same criteria, PKD formed complexes only very weakly with PKCε, and did not bind PKCθ. When PKCζ was coexpressed with PKD mutants containing either complete or partial deletions of the PH domain, both PKCζ immunoreactivity and PKC activity in PKD immunoprecipitates were sharply reduced. In contrast, deletion of an amino-terminal portion of the molecule, either cysteine-rich region, or the entire cysteine-rich domain did not interfere with the association of PKD with PKCζ. Furthermore, a glutathione S-transferase-PKDPH fusion protein bound preferentially to PKCζ. These results indicate that the PKD PH domain can discriminate between closely related structures of a single enzyme family, e.g. novel PKCs ε and ζ, thereby revealing a previously undetected degree of specificity among protein-protein interactions mediated by PH domains.

Protein kinase C (PKC), a major cellular target for the potent tumor-promoting phorbol esters (1, 2), has been implicated in the mediation of diverse cellular functions, including short-term regulation of ion fluxes, receptor ligand binding, and signal transduction, and a wide range of longer term effects including proliferation, differentiation, transformation, and regulation of the mammalian cell cycle (3–6). At least 10 PKC isoforms, i.e. the classic (α, β1, β2, and γ), novel (δ, ε, η, and θ), and atypical PKCs (ζ, λ) have been identified by molecular cloning techniques (5, 7, 8). The different PKC isoforms exhibit distinct patterns of expression in different cell types and tissues as well as distinct subcellular distributions, leading to the view that they play distinct, rather than redundant roles in signal transduction (5, 9, 10). However, since very few of their specific substrates and binding partners have been identified, it has not yet been possible to ascribe to each isoform a unique cellular function.

The recently identified protein kinase D (PKD) is a mouse serine protein kinase with distinct structural and enzymological properties (11–13). In particular, the catalytic domain of PKD, which is distantly related to Ca2+-regulated protein kinases (11), possesses only a low degree of sequence similarity to the highly conserved regions of the kinase subdomains of the PKC family (14). Accordingly, PKD does not phosphorylate a variety of substrates utilized by PKCs indicating that PKD is a protein kinase with distinct substrate specificity (11, 12, 15). The amino-terminal region of PKD contains a tandem repeat of cysteine-rich regions that bind phorbol esters and diacylglycerol (11), similar to those found in classical and novel PKCs (16). However, unlike all PKCs, PKD does not possess a regulatory pseudosubstrate domain upstream of the first cysteine-rich motif. An additional structural feature that distinguishes PKD from the PKC family is the presence of a PH domain, interposed between the second cysteine-rich motif and the catalytic domain. PH domains are modular protein domains which mediate protein-protein and lipid-protein interactions and are found in many cytoskeletal and signal transducing proteins (17, 18). The PKD PH domain has been found to contribute to regulation of PKD catalytic activity (15).

Recently we reported that exposure of intact cells to biologically active phorbol esters, membrane-permeant diacylglycerol or bryostatin-1 induces PKD activation via a PKC-dependent pathway (19, 20). PKD activated within cells via PKC can be immunopurified from cell extracts in an active state that is independent of exogenously added cofactors (19). Growth factors and mitogenic neuropeptides that promote phospholipid turnover and diacylglycerol generation also stimulate this pathway, indicating that PKD activation is an early event in cell stimulation (21). We further substantiated the role of PKCs in PKD activation by coexpression of PKD together with constitutively active mutant PKC isoforms. These studies revealed that novel PKCζ and PKCη fully activated PKD within cells, whereas classical PKCβ1 or atypical PKCζ did not (19). These results raise the possibility that PKD functions downstream of novel PKCs in a previously undescribed signal transduction pathway.

Here, we demonstrate, for the first time, that coexpression of...
PKD with PKCζ in COS-7 cells leads to the formation of a stable PKD-PKCζ complex. Strikingly, we found very little evidence of complex formation between PKD and the PKCζ isoform despite its close similarity to PKCζ, and no evidence for a stable interaction between PKD and PKCα. Our results also demonstrate that the PH domain is critical for stable PKD-PKCζ complex formation, thus indicating that these domains can mediate highly selective protein-protein interactions.

**Experimental Procedures**

**Cell Culture and Transfections—**COS-7 cells were maintained by subculture in 10-cm tissue culture plates, every 3–4 days in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 10% CO2. For experimental dishes, confluent cells were subcultured at a density of 6 × 10^6 cells/ml in 6- or 10-cm dishes on the day prior to transfection. All transfections and co-transfections were carried out with equivalent amounts of DNA (6 μg/6-cm dish, 12 μg/10-cm dish), using vector pcDNA3 as the control DNA added to single transfections. Transfections were carried out in Opti-MEM (Life Technologies, Inc.) using Lipofectin (Life Technologies, Inc.) at 10 μg/6-cm dish or 20 μg/10-cm dish, added to cells in a final volume of 2.5 ml/6-cm dish or 5 ml/10-cm dish, following formation of DNA-Lipofectin complexes according to the protocol provided by the manufacturer. Cells were allowed to take up complexes in the absence of fetal bovine serum for 5–6 h or overnight, then fetal bovine serum (10% final concentration) in Opti-MEM was added to the dishes to yield a final volume of 5 ml/6-cm dish or 10 ml/10-cm dish. Cells were used for experiments after a further 48–72 h of incubation.

**cDNA Constructs used in Transfections—**The constructs pcDNA3-PKD encoding PKD (12), pcDNA3-PKD/K631M encoding kinase-deficient mutant PKD (19), cysteine-rich domain mutant constructs pcDNA3-PKD/Cys1, pcDNA3-PKDCys2, and pcDNA3-PKD/Cys2 (22), the PKD mutant pcDNA3-PKDΔNH₂ with a deletion of the NH₂-terminal first 66 residues including the putative transmembrane motif (22) and the PK domain mutant pcDNA3-PKDΔPH lacking the entire PH domain, pcDNA3-PKDΔ1–4β lacking the first 4 β-strands of the 7-stranded β-barrel portion, and pcDNA3-PKDΔα lacking the COOH-terminal α-helix have been described previously (15). An additional PH domain mutant PKD construct, pcDNA3-PKDΔ1–7β, encoding PKD lacking all seven β-strands of the β-barrel (deletion of amino acids 429–532) was constructed using PCR, as follows: primers carrying the Axl site (forward primer, 5'-GTCAGCGCGCGCCCGGAGG-3', reverse primer, 5'-GTCAGCGCGCCCGCCACAGGGAGGCT-3') were used to amplify the COOH-terminal part of the PH domain (from Asp333 to Gly557). The generated PCR product was Axl-digested and reinserted into the Axl site of pcDNA3-PKDΔPH, resulting in the plasmid pcDNA3-PKDΔ1–7β. The cDNAs encoding wild-type and active mutants were kindly gifted by Dr. Peter Parker from the Cancer Research Fund, and have been described previously (21).

**Preparation of PKD PH Domain Fusion Protein—**The cDNA sequence spanning the entire PKD domain (aa 418–567) was amplified by PCR from wild-type PKD using specific oligonucleotide primers (forward primer, 5'-GTCAGCGCGCGCCCGGAGG-3', reverse primer, 5'-GTCAGCGCGCGCCCGCCACAGGGAGGCT-3') containing restriction sites for BamHI and EcoRI, respectively (underlined). The resulting PCR product was subcloned as a BamHI-EcoRI fragment into the vector pGEX4T3 (Pharmacia Biotech Inc.) to generate the bacterial expression construct pGEX-GST-PKDPH. The 42-kDa GST-PKD PH domain fusion protein (GST-PKDPH) was expressed in *Escherichia coli*, purified on glutathione-agarose beads, eluted with 25 mM reduced glutathione, dialyzed against phosphate-buffered saline, and stored at −20 °C in 40% glycerol. Purity and concentration of the recombinant protein were assessed by SDS-PAGE and Coomasie Brilliant Blue staining.

**Assays of PKG-PKT-PKDPH Fusion Protein Binding in Vitro—**COS-7 cells transiently transfected with the different PKC isoforms were lysed 72 h after transfection by removal of growth medium from cells on ice and addition of lysis buffer (50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM dithiothreitol, 10 mM/mg protein, 100 μg/ml leupeptin, 1 mM AEBSF (Pefabloc), and 1% Triton X-100), and the resulting extracts were combined with either GST (control) or GST-PKDPH fusion proteins preadsorbed onto glutathione-agarose beads. After 2 h at 4 °C, the complexes were washed 8 times with lysis buffer, and bound proteins were extracted with SDS-PAGE sample buffer and subjected to SDS-PAGE and Western analysis using isof orm-specific antisera to detect associated PKC, as described in figure legends.

**Immunoprecipitations—**COS-7 cells transfected with wild-type or mutant PKD or co-transfected together with different PKC isoforms were lysed as described above. Small amounts (typically 1/10) of these extracts were preincubated with SDS-PAGE sample buffer (1 ml Tris-HCl, pH 8.6, 8% SDS, 0.5 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol) for Western blot analysis. PKD was immunoprecipitated at 4 °C for 3 h with either the PA-1 antiserum (1:100 dilution) raised against the synthetic peptide EEKMKALS-ERVISL that corresponds to the predicted COOH-terminal region of PKD, as described previously (12), or a 1:200 dilution of a commercial antiserum (PKD C-20, Santa Cruz Biotechnologies), which also recognizes the COOH-terminal region of PKD. PKCs were immunoprecipitated using respective PKC antisera at 1:1000 dilution. Immune complexes were recovered using protein A coupled to agarose.

**In Vitro Kinase Assays—**Immunocomplexes were washed twice with lysis buffer, then twice with kinase buffer consisting of 30 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol. Autophosphorylation reactions were initiated by combining 20 μl of immunoprecipitates with 10 μl of a phosphorylation mixture containing [γ-32P]ATP (3 μCi/reaction diluted with cold ATP to give a final concentration of 100 μM) in kinase buffer (final reaction volume, 30 μl), and transferred subject to autoradiography to visualize radiolabeled protein bands. For assays of exogenous substrate phosphorylation, immune complexes were processed as for autophosphorylation reactions, then substrates (either syntide-2 or ε-peptide at final concentrations 2.5 or 1.75 mg/ml, respectively) were added in the presence of [γ-32P]ATP (2 μCi/reaction diluted with cold ATP to give a final concentration of 100 μM) in kinase buffer (final reaction volume, 30 μl) and transferred to a water bath at 30 °C for 10 min, then terminated by addition of 1 ml of ice-cold kinase buffer and removed to an ice bucket. Immune complexes were recovered by centrifugation, and the proteins were extracted for SDS-PAGE analysis by incubating SDS-PAGE sample buffer (2.5×) with the immune complexes and subjected to autoradiography to visualize radiolabeled protein bands.

**Western Blot Analysis—**For Western blot analysis, immune complexes and proteins associated with glutathione-agarose/GST fusion protein complexes were washed as for *in vitro* kinase reactions, then extracted by boiling in SDS-PAGE sample buffer. Samples of cell lysates were directly solubilized by boiling in SDS-PAGE sample buffer. Following SDS-PAGE on 8% gels, proteins were transferred to Immobilon-P membranes (Millipore) as described previously (21) and blocked by overnight incubation with 5% non-fat dried milk in phosphate-buffered saline, pH 7.2. Membranes were incubated at room temperature for 2 h with antisera specifically recognizing either PKD or the different PKC isoforms, at a dilution of 1 μg/ml, in phosphate-buffered saline containing 3% non-fat-dried milk. Immunoreactive bands were visualized using either horseradish peroxidase-conjugated anti-rabbit IgG and subsequent enhanced chemiluminescence detection or 125I-labeled protein A followed by autoradiography.

**Materials—**[γ-32P]ATP (6000 Ci/mmol) was from Amersham International (United Kingdom). Protein A-agarose and AEBSF (Pefabloc) were from Boehringer Mannheim (UK). Antisera (PKD C-20, PKCζ C-20, PKCs C-15, PKCγ C-15, and PKCβ C-15) used in Western blot analysis were from Santa Cruz Biotechnologies, Palo Alto, CA. PKCα and PKCδ reagents were from Calbiochem, Opti-MEM and Lipofectin were from Life Technologies, Inc. Glutathione-Sepharose was from Pharmacia Biotech. Syntide-2 peptide and immunizing peptide EEKMKALS-ERVISL corresponding to the PKD COOH terminus were synthesized at the Imperial Cancer Research Fund. ε-PEptide was from Alexis Biochemicals. All other reagents were from standard suppliers or as described in the text and were the highest grade commercially available.

**Results**

An 80-kDa Phosphoprotein Associates with PKD in Cells Coexpressing PKD and PKCζ—COS-7 cells transiently transfected with pcDNA3-PKD either alone or together with PKCζ, PKCκ, or PKCξ expression constructs encoding constitutively active PKC mutants were incubated with or without 200 nM PDBu for 10 min and lysed. PKD was immunoprecipitated from the extracts and the resulting immunoprecipitates were sub-
PKD Interacts Preferentially with PKCη

**Fig. 1. Detection of PKD-PKCη complexes within PKD immunoprecipitates by in vitro kinase assays.** A, exponentially growing COS-7 cells (40–60% confluent) were co-transfected with pcDNA3-PKD (PKD) together with either empty vector pcDNA3 (−) or the vectors containing the cDNAs encoding the constitutively active PKC isoforms, as indicated. At 72 h post-transfection, the cultures were either treated with 200 nM PDBu (PDB) (+) or left untreated (−) for 10 min. The cultures were then lysed and the lysates immunoprecipitated with the PA-1 antiserum and further analyzed by in vitro kinase reactions as described under “Experimental Procedures.” A representative autoradiogram is shown. Similar results were obtained from each of at least three similar experiments. B, COS-7 cells were co-transfected with pcO2-PKCη plasmid encoding the wild-type PKCη (+) together with either pcDNA3-PKD or empty vector pcDNA3, as indicated (−). After 72 h of incubation, the cultures were lysed and the lysates immunoprecipitated with the commercially obtained anti-PKD antiserum in the absence (−) or presence (+) of the immunizing peptide (Imm Pep) used to generate the PA-1 antiserum at a concentration of 20 μg/ml lysate, and further analyzed by autoradiography reactions. This experiment was repeated twice with similar results. A representative autoradiogram is shown. C, left, COS-7 cells were co-transfected with pcDNA3-PKD (PKD), pcDNA3-PKDK618M (KM), or empty vector pcDNA3 (−) together with the plasmid construct encoding constitutive active PKCη (η*), as indicated. At 72 h post-transfection, the cultures were lysed and the lysates immunoprecipitated with the PA-1 antiserum and further analyzed by in vitro kinase reactions. This experiment was repeated three times with similar results. A representative autoradiogram is shown. C, right, COS-7 cells were co-transfected with either pcDNA3-PKD (PKD) or pcDNA3-PKDK618M (KM) together with plasmid constructs encoding wild-type PKCη (η), active mutant PKCη (η*), or empty vector pcDNA3 (−), as indicated. At 72 h post-transfection, the cultures were either treated with 200 nM PDBu (+) or left untreated (−) for 10 min, as indicated. The cultures were then lysed and the lysates immunoprecipitated with the PA-1 antiserum and further analyzed by exogenous substrate phosphorylation assays using η peptide as described under “Experimental Procedures.”

Initially, we considered whether this band might be the result of either nonspecific immunoprecipitation by the PA-1 antiserum or long-term overexpression of the active mutant PKCη. To test this, we used a commercially available antiserum (rather than PA-1) to perform PKD immunoprecipitations from cells coexpressing PKD together with the wild-type PKCη, followed by in vitro kinase assays. As shown in Fig. 1B, this assay also led to the isolation of immune complexes containing 110- and 80-kDa phosphoproteins. Thus, it was possible to substitute either the primary antibody used in the immunoprecipitation or the wild-type for the active mutant PKCη and still retain both bands. In addition, both autophosphorylated PKD and the coprecipitated 80-kDa phosphoprotein band were eliminated when the immunoprecipitation reactions were carried out in the presence of the immunizing peptide used to generate the PA-1 antiserum (Fig. 1B).

To test whether the 80-kDa band represented a proteolytic fragment of autophosphorylated PKD, we co-transfected an expression construct, pcDNA3-PKD/K618M, which encodes a kinase-deficient mutant of PKD, together with PKCη, and performed immunoprecipitations with the PA-1 antiserum followed by in vitro kinase assays. As shown in Fig. 1C, this assay again resulted in the appearance of the 80-kDa band. Importantly, whereas the intensity of this band was similar to that seen when the wild-type PKD was used for co-transfection, the intensity of the 110-kDa band was drastically reduced in comparison with that generated by the co-transfection with wild-
PKD Interacts Preferentially with PKC-9277

PKD interacts preferentially with PKC isoforms by Western blotting. Exponentially growing COS-7 cells were either co-transfected with empty vector pcDNA3 (−) or pcDNA3-PKD (A) or co-transfected with vectors containing the cDNAs encoding either wild-type (ε and η; C, D, and G) or constitutively active PKC isoforms (ε, ε*, and η*; B, E, and F) together with either pcDNA3-PKD (+) or empty vector pcDNA3 (−), as indicated. At 72 h post-transfection, the cultures were lysed and the lysates were immunoprecipitated with either PA-1 antiserum (A-F) or the PKC-9277 antisera (G). After washing, the immune complexes were either extracted with gel loading buffer and analyzed by SDS-PAGE followed by Western blot analysis using the isoform-specific polyclonal antisera against either the different PKCs (A-F) or PKD (G, PKD W. Blot) or analyzed by in vitro kinase assays (G, IVK), as indicated. Arrows indicate the position of PKD in anti-PKC immunoprecipitates (G) and PKC-9277 in PKD immunoprecipitates (D and F). Similar results were obtained in at least three experiments.

Type PKD2 (Fig. 1C, left). These data also indicate that the kinase activity of PKD is not required for the association with the 80-kDa phosphoprotein.

PKC-9277 Specifically Associates with PKD—Previous studies indicated that ε-peptide, a peptide based on the pseudosubstrate domain of PKCε, is a substrate for all PKCs (24), but is a poor substrate for PKD (11, 12, 15). In agreement with these studies, anti-PKD immunoprecipitates from lysates of cells transfected with PKD (or the kinase-deficient mutant, PKD/K618M) did not phosphorylate ε-peptide, even when PKD was activated within cells by PDBu treatments (Fig. 1C, right). Surprisingly, PKD immunoprecipitates from lysates of cells co-transfected with PKD (either wild-type or PKD/K618M) and PKC-9277 (either constitutive active or wild-type) contained activity which strongly phosphorylated ε-peptide. Since this activity was associated with both catalytically active and inactive forms of PKD, and PKD does not phosphorylate the peptide, we conclude that the activity was due to an associated protein kinase (e.g., contributed by the 80-kDa phosphoprotein) whose activity did not require the catalytic activity of PKD.

Since PKC-9277 has an apparent molecular mass of approximately 80 kDa in SDS-PAGE, and since the 80-kDa phosphoprotein had appeared only when PKD was coexpressed together with mutant or wild-type PKC-9277, it seemed plausible that coexpression of PKD and PKC-9277 resulted in the formation of a PKD-PKC-9277 complex that persists during immunoprecipitation of PKD. To test this hypothesis directly, we used Western blot analysis to examine whether PKC-9277 was present in PKD immunoprecipitates. In view of the results indicating that the 80-kDa band is not detected in PKD immunoprecipitates from lysates of cells co-transfected with PKD together with either PKCε or PKCζ (Fig. 1A), we also performed Western blot analysis to assess whether these isoforms were present in PKD immunoprecipitates. Since PKCε is abundantly expressed in COS-7 cells, we also tested for the presence of this isoform in PKD immunoprecipitates. Lysates of cells either transfected with PKD or co-transfected with PKD and PKCs ε, η, or ζ were either examined directly by Western blot analysis using polyclonal antibodies that specifically recognize PKCs ε, ζ, ε, or η or subjected to immunoprecipitation reactions with PA-1 antiserum followed by Western analysis. As shown in Fig. 2, D and F, anti-PKC-9277 immunoblotting of PKD immunoprecipitates indicated that both wild-type and mutant PKCζ associated with PKD. Similar analysis did not detect the presence of PKCs ε, ζ, or ε (either wild-type or constitutively active) in PKD immunoprecipitates from the corresponding cell lysates (Fig. 2C and E). However, longer exposure of the autoradiograms did reveal a faint band of anti-PKCs immunoreactivity (not shown), indicating that PKCζ may also associate with PKD but to a much lesser degree.

Although the experiments in Figs. 1 and 2 indicate that PKC-9277 was present in PKD immunoprecipitates from cells co-transfected with these two proteins, these results were obtained using antibodies directed against PKD. To substantiate further the existence of a PKD-PKC-9277 interaction, we also performed immunoprecipitations using an antibody directed against the opposite partner in the association, i.e. PKCζ, followed by Western analysis to detect the interacting PKD protein. As illustrated in Fig. 2G, in vitro kinase assays revealed the presence of an autophosphorylated band in the position corresponding to PKD (110 kDa) that depended on the presence of co-transfected PKD. Furthermore, Western blot analysis revealed the presence of immunoreactive PKD when both proteins were co-transfected. Thus, the results shown in Fig. 2G demonstrate the presence of PKD in PKC-9277 immunoprecipitates from lysates of cells co-transfected with PKD and PKCζ.

Phosphorylation of PKC and PKD Substrates by PKD Immunoprecipitates—To further address the specificity of the PKD-PKCζ interaction, we measured phosphorylation of exogenous substrates by PKD immunoprecipitates from cells expressing each PKD-PKCζ combination. In initial experiments, we examined phosphorylation of ε-peptide by endogenously expressed and transfected PKC isoforms in anti-PKC immunoprecipi-
PKC Interacts Preferentially with PKCζ

Determination of PKD binding specificity toward different PKC isoforms by phosphorylation of exogenous substrates. Exponentially growing COS-7 cells were transfected with either empty vector pcDNA3 (−) or plasmid constructs encoding the constitutively active PKC isoforms, or co-transfected with pcDNA3-PKD together with either empty vector or the active PKC isoforms, as indicated. At 72 h post-transfection, the cultures were lysed, the lysates were immunoprecipitated with the PA-1 antiserum, and subjected to exogenous substrate phosphorylation assays as described under “Experimental Procedures.” A and B, lower panel, ε-peptide phosphorylation assay. B, upper panel, syntide-2 phosphorylation assay. The results are from at least three experiments, each performed in duplicate (B), or from a single experiment performed in triplicate (A).

For assays of PKD immunoprecipitates we used both ε-peptide, a poor substrate for PKD (Fig. 1), and syntide-2 (25, 26), a synthetic peptide previously demonstrated to be an excellent substrate for PKD (11), thereby exploiting the distinct substrate specificity of PKCs and PKD. In this way, we measured both PKD activation and the retention of PKC activity bound to PKD in the same immune complexes. In agreement with previous results, when the PA-1 antiserum was used to immunoprecipitate PKD from cells overexpressing only PKD, syntide-2 assays revealed a low basal activity that was dramatically increased upon PDBu stimulation of cells (Fig. 3B, upper graph). Again, these immunoprecipitates did not phosphorylate ε-peptide to any significant extent, even after the cells had been stimulated with PDBu (Fig. 3B, lower graph). In contrast with these results, when PKD was immunoprecipitated from cells co-transfected with PKD and either mutant or wild-type PKCζ, both syntide-2 and ε-peptide were strongly phosphorylated, indicating the presence of both PKD and PKCζ enzyme activities. These results extend those of Fig. 1 by providing evidence that PKCζ activity, and not that of PKCζ or PKCe, is preferentially associated with PKD. Thus, phosphorylation of the PKCζ peptide, an excellent PKC substrate, correlates with the appearance of the 80-kDa band in the in vitro kinase assays (Fig. 1) and with the detection of immunoreactive PKCζ in PKD immunoprecipitates (Fig. 2).

Interestingly, in cells co-transfected with PKD and PKCe, phosphorylation of syntide-2 was also nearly maximal even in the absence of PDBu stimulation even though phosphorylation of ε-peptide was only slightly above control levels. This result demonstrated that, in agreement with results in Figs. 1 and 2, PKCe had activated PKD during coexpression but is retained in the PKD immune complexes only slightly, implying that the involvement of PKCs in PKD activation is mediated by a transient event rather than, or in addition to, the PKD-PKC association itself. As expected, immunoprecipitates from cells co-transfected with PKD and PKCζ phosphorylated syntide-2 in a manner indistinguishable from that from cells expressing only PKD, and did not phosphorylate ε-peptide. These results confirm that PKD preferentially forms complexes with PKCζ rather than with PKCe, and does not form complexes with PKCε.

The PKD PH Domain Is Required for Formation of a PKD-PKCζ Complex—Recent reports have shown that PH domains within the Bruton’s tyrosine kinase and the serine-threonine kinase PKB/Akt can mediate association of these proteins with multiple isoforms of PKC (27, 28). In contrast, our results demonstrated that PKD preferentially associates with PKCζ. To examine whether the PH domain of PKD could mediate this specific association of PKD with PKCζ, we co-transfected COS-7 cells with wild-type PKCζ together with expression constructs encoding either intact PKD, PKD lacking the entire PH domain, or PKD with the PH domain truncated by partial deletions (15). PKD immunoprecipitates from these cells were subjected to Western blot analysis and phosphorylation of ε-peptide to assess the presence of PKCζ in the immune complexes (Fig. 4, A and B). In agreement with data shown in Fig. 2, wild-type PKCζ was co-immunoprecipitated with intact PKD (Fig. 4B). Similarly, these immunoprecipitates contained PKC activity, as revealed by ε-peptide assays (Fig. 4A). In contrast, when PKCζ was coexpressed with any of the PKD mutants containing either complete or partial deletions of the PH domain, both PKC activity (Fig. 4A) and PKCζ immunoreactivity (Fig. 4B) in PKD immunoprecipitates were sharply reduced. Control Western blots demonstrated that PKCζ was expressed in each transfection in similar amounts, as was each of the wild-type or mutant PKD proteins (Fig. 4B).

In order to determine the specificity of inhibition of PKD-PKCζ interaction by deletion of the PKD PH domain, we also examined the effect of other deletion mutants of PKD on the PKD-PKCζ interaction. COS-7 cells co-transfected with PKCζ together with PKD mutants lacking either a portion of the amino terminus containing the hydrophobic sequence of PKD, the first or second cysteine-rich regions, or the entire tandem

![Graph](image-url)

**Fig. 3.** Determination of PKD binding specificity toward different PKC isoforms by phosphorylation of exogenous substrates. Exponentially growing COS-7 cells were transfected with either empty vector pcDNA3 (−) or plasmid constructs encoding the constitutively active PKC isoforms, or co-transfected with pcDNA3-PKD together with either empty vector or the active PKC isoforms, as indicated. At 72 h post-transfection, the cultures were lysed, the lysates were immunoprecipitated with the PA-1 antiserum, and subjected to exogenous substrate phosphorylation assays as described under “Experimental Procedures.” A and B, lower panel, ε-peptide phosphorylation assay. B, upper panel, syntide-2 phosphorylation assay. The results are from at least three experiments, each performed in duplicate (B), or from a single experiment performed in triplicate (A).
PKD interacts preferentially with PKC\(\eta\).

PKD interacts preferentially with PKC\(\eta\). COS-7 cells transiently transfected with active mutant PKC\(\xi\) (\(\xi^+\)), wild-type PKC\(\epsilon\) (\(\epsilon\)), wild-type PKC\(\eta\) (\(\eta\)), or active mutant PKC\(\eta\) (\(\eta^+\)) were lysed after 72 h of incubation. Equivalent amounts of cell lysates were combined with immobilized GST or GST-PKDPH, as indicated, and then processed by incubations, washing procedures, and Western blot analysis as described under "Experimental Procedures." A, GST or GST-PKDPH (1 \(\mu\)g each) was incubated with lysates from cells transfected with active mutant PKC\(\xi\) (\(\xi^+\)), wild-type PKC\(\epsilon\) (\(\epsilon\)), wild-type PKC\(\eta\) (\(\eta\)), or active mutant PKC\(\eta\) (\(\eta^+\)) prior to further processing. B, upper panels, different amounts of standard PKC \(\epsilon\) and \(\eta\) proteins, as indicated, were subjected to Western blot analysis using the isoform-specific antisera. Middle panels, lysates were prepared from cells transfected with either PKC\(\eta\) or PKC\(\epsilon\) and subjected, in parallel, to Western blot analysis using the indicated amounts of cell lysates (diluted 1:1 with 2 \(\times\) SDS-PAGE gel loading buffer), as indicated. Lower panels, GST (10 \(\mu\)g) or different amounts of GST-PKDPH (100 ng, 300 ng, 1 \(\mu\)g, 3 \(\mu\)g, 10 \(\mu\)g and 15 \(\mu\)g) were preadsorbed to glutathione-agarose beads, combined with lysates from cells transfected with either wild-type PKC\(\eta\) or PKC\(\epsilon\), as indicated, and then incubated and further processed as in A. Results shown are representative of at least three independent experiments.

**Fig. 4.** Role of the PKD PH domain in mediating PKD-PKC\(\eta\) complex formation. A, exponentially growing COS-7 cells were transfected with either pcDNA3-PKD (open bars) or co-transfected with PKD\(\Delta PH\) lacking the entire PH domain (\(\Delta PH\)), PKD\(\Delta 1–4\beta\) lacking the first amino-terminal four \(\beta\)-sheets (\(\Delta 4\beta\)), PKD\(\Delta 1–7\beta\) lacking all seven \(\beta\)-sheets (\(\Delta 7\beta\)), or PKD\(\Delta \alpha 1\) lacking the carboxyl-terminal \(\alpha\)-helix (\(\Delta \alpha 1\)), as indicated, together with wild type PKC\(\eta\) (filled bars). At 72 h post-transfection, the cultures were lysed, the lysates were immunoprecipitated with the PA-1 antiserum, and subjected to Western blot analysis using the indicated amounts of cell lysates (diluted 1:1 with 2 \(\times\) SDS-PAGE gel loading buffer), as indicated. Lower panels, GST (10 \(\mu\)g) or different amounts of GST-PKDPH (100 ng, 300 ng, 1 \(\mu\)g, 3 \(\mu\)g, 10 \(\mu\)g and 15 \(\mu\)g) were preadsorbed to glutathione-agarose beads, combined with lysates from cells transfected with either wild-type PKC\(\eta\) or PKC\(\epsilon\), as indicated, and then incubated and further processed as in A. Results shown are representative of at least three independent experiments.

**Fig. 5.** Binding of different PKCs to the GST-PKDPH fusion protein. COS-7 cells transiently transfected with active mutant PKC\(\xi\) (\(\xi^+\)), wild-type PKC\(\epsilon\) (\(\epsilon\)), wild-type PKC\(\eta\) (\(\eta\)), or active mutant PKC\(\eta\) (\(\eta^+\)) were lysed after 72 h of incubation. Equivalent amounts of cell lysates were combined with immobilized GST or GST-PKDPH, as indicated, and then processed by incubations, washing procedures, and Western blot analysis as described under "Experimental Procedures." A, GST or GST-PKDPH (1 \(\mu\)g each) was incubated with lysates from cells transfected with active mutant PKC\(\xi\) (\(\xi^+\)), wild-type PKC\(\epsilon\) (\(\epsilon\)), wild-type PKC\(\eta\) (\(\eta\)), or active mutant PKC\(\eta\) (\(\eta^+\)) prior to further processing. B, upper panels, different amounts of standard PKC \(\epsilon\) and \(\eta\) proteins, as indicated, were subjected to Western blot analysis using the isoform-specific antisera. Middle panels, lysates were prepared from cells transfected with either PKC\(\eta\) or PKC\(\epsilon\) and subjected, in parallel, to Western blot analysis using the indicated amounts of cell lysates (diluted 1:1 with 2 \(\times\) SDS-PAGE gel loading buffer), as indicated. Lower panels, GST (10 \(\mu\)g) or different amounts of GST-PKDPH (100 ng, 300 ng, 1 \(\mu\)g, 3 \(\mu\)g, 10 \(\mu\)g and 15 \(\mu\)g) were preadsorbed to glutathione-agarose beads, combined with lysates from cells transfected with either wild-type PKC\(\eta\) or PKC\(\epsilon\), as indicated, and then incubated and further processed as in A. Results shown are representative of at least three independent experiments.

cysteine-rich domain (22) were lysed and immunoprecipitated either with the PA-1 antiserum or with the PKC\(\eta\) antiserum. As shown in Fig. 4C, Western analysis of each of these reciprocal immunoprecipitates revealed the presence of the opposite binding partner in the association. Similar to the results shown in Fig. 4, A and B, deletion of the PH domain prevented the detection of both binding partners in the respective immunoprecipitates (Fig. 4C). Taken together, these data indicate that the PH domain of PKD is necessary for efficient complex formation with PKC\(\eta\). However, it appears that the PH domain is not the only determinant of binding to PKD, as some PKC\(\eta\) immunoreactivity and activity was associated with immunoprecipitates with the PH domain of PKD. The recovered immunoprecipitates were probed using the anti-PKC\(\eta\) antiserum, and PKC\(\eta\) immunoprecipitates were probed using the anti-PKD antiserum, as indicated. The arrow indicates the expected position for PKD\(\Delta PH\) protein.
PKD Interacts Preferentially with PKC\(\eta\)

...ity to associate with the GST-PKDPH fusion protein. In contrast, much less PKCe was recovered from parallel cell lysates with this fusion protein. Control Western blot analysis of cell lysates confirmed that PKCe was indeed overexpressed in these cells, allowing us again to infer that there was a preferential binding of PKD to PKC\(\eta\) over PKCe. To address this issue more quantitatively, we performed Western blot analysis using different amounts of transfected cell lysates and purified protein standards (PKCe and PKC\(\eta\)) to determine the absolute amounts of each PKC isoform produced in cell lysates and recovered by the fusion protein. As determined from data shown in Fig. 5B, each PKC isoform was present in the cell lysates in similar amounts (approximately 1.5 \(\mu\)g/ml for each PKC in the initial lysates). We then examined the binding of GST-PKDPH to PKCe and PKC\(\eta\) in cell lysates as a function of the fusion protein concentration. These experiments demonstrated that the fusion protein bound to PKC\(\eta\) at a concentration as low as 0.1 \(\mu\)g/ml. In contrast, very little PKCe was bound to the fusion protein even at a 100-fold higher concentration (Fig. 5B).

**DISCUSSION**

Our previous studies have shown that PKD is activated in vivo by treatment with biologically active phorbol esters or multiple agonists via a PKC-dependent pathway (19–21). The results presented here demonstrate the formation of a complex between PKC\(\eta\) and PKD and thus, identify a new aspect of the relationship between these two enzymes. Although further studies will be required to address the exact physiological role of this complex, the association of PKD and PKC\(\eta\) could play a part in the regulation of the activity and/or subcellular localization of this enzyme. Indeed, recent findings from Cantley and co-workers (29) indicated that transfected PKD/PKC\(\mu\) complexed with endogenous phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase enzymes present in COS-7 cells. Importantly, truncation mutants of PKD/PKC\(\mu\) lacking a portion of the molecule between the NH\(_2\)-terminal hydrophobic region and the PH domain failed to retain the binding to the lipid kinases (29). In view of the results presented here, we conclude that different domains of the regulatory region of the PKD molecule are involved in mediating interactions with different proteins. Together, these results suggest the attractive possibility that PKD/PKC\(\mu\) may act as a scaffold protein, through its different domains, promoting the assembly of signaling enzymes.

From our results, it is clear that the PH domain of PKD mediates a major portion of the binding to PKC\(\eta\), other (even larger) deletions of the PKD molecule being without effect. Recently, two important signaling proteins, PKB/Akt and BTK, have been shown to interact via their PH domains with multiple isoforms of PKC. In fact, the fusion protein containing only the PKD PH domain to PKC\(\eta\) requires a region within the C1 regulatory domain in the vicinity of the pseudosubstrate domain of this enzyme (31). We find that PKD associates with an active PKC\(\eta\) mutant with a deletion from amino acids 155 to 171 within its pseudosubstrate domain (as well as with wild-type PKC\(\eta\)). Therefore, the interaction of the PKD PH domain with PKC\(\eta\) does not require the pseudosubstrate portion of the molecule.

Recently, a model for the binding of PH domains to proteins has been presented (32) in which a candidate sequence (HIK\(^x\)E), identified by sequence homology analysis, was proposed to act as a target for the PH domains. However, the putative binding sequence HIK\(^x\)E is present in both PKC\(\eta\) and PKC\(\zeta\) and consequently is unlikely to play a critical role in determining the differential binding of the PH domain of PKD to different PKCs found in the present study.

In conclusion, our findings demonstrate that coexpression of PKD with PKC\(\eta\) leads to the formation of a stable PKD-PKC\(\eta\) complex. Strikingly, we found very little evidence of complex formation between PKD and the PKCe isoform despite its close similarity to PKC\(\eta\), and no evidence for a stable interaction between PKD and PKC\(\zeta\). Our results also demonstrate that the PH domain is critical for stable PKD-PKC\(\eta\) complex formation.

We conclude that the PKD PH domain can discriminate between closely related structures of a single enzyme family, e.g., novel PKCs \(\epsilon\) and \(\eta\), thereby revealing a previously undetected degree of specificity among protein-protein interactions mediated by PH domains.

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