Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation

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Abstract Protein kinase D (PKD)/protein kinase Cμ (PKCμ), a serine/threonine protein kinase with distinct structural and enzymological properties, is rapidly activated in intact cells via PKC. The amino-terminal region of PKD contains a cysteine-rich domain (CRD) that directly binds phorbol esters with a high affinity. Here, we show that treatment of transfected RBL 2H3 cells with phorbol 12,13-dibutyrate (PDB) induces a striking CRD-dependent translocation of PKD from the cytosol to the plasma membrane, as shown by real time visualization of a functional green fluorescent protein (GFP)-PKD fusion protein. A single amino acid substitution in the second cysteine-rich motif of PKD (P287G) prevented PDB-induced membrane translocation but did not affect PKD activation. Our results indicate that PKD translocation and activation are distinct processes that operate in parallel to regulate the activity and localization of this enzyme in intact cells.

Key words: Cysteine-rich domain; Membrane localization; Protein kinase C; Phorbol ester

1. Introduction

Protein kinase C (PKC), a major target for the tumor promoting phorbol esters, has been implicated in the signal transduction of a wide range of biological responses, including changes in cell morphology, differentiation and proliferation [1,2]. Molecular cloning has demonstrated the presence of multiple related PKC isoforms [2–5], i.e. conventional PKCs (α, β_1, β_2 and γ), novel PKCs (δ, ε, η and θ) and atypical PKCs (ζ and λ), all of which possess a highly conserved catalytic domain.

Protein kinase D (PKD)/PKCμ is a serine/threonine protein kinase with distinct structural, enzymological and regulatory properties [6,7]. The catalytic domain of PKD is distantly related to Ca^{2+}-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family [8]. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs, indicating that PKD is a protein kinase with a distinct substrate-specificity [6,9]. In contrast to all PKCs, the NH_2-terminal region of PKD contains a pleckstrin homology (PH) domain that regulates enzyme activity [10] and lacks a sequence with homology to a typical PKC auto-inhibitory pseudosubstrate motif [6]. In addition, the amino-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs [6,7] that binds phorbol esters with high affinity [6,11]. The two motifs within this cysteine-rich domain (CRD) are not functionally equivalent, however, with the second cysteine motif acting as the major site for phorbol ester binding, both in vitro and in vivo [11]. PKD and its human homologue PKCμ can be stimulated in vitro by either bioactive phorbol esters or diacylglycerol (DAG) in the presence of phosphatidylserine [12,13], indicating that PKD/PKCμ is a phorbol ester/DAG-stimulated kinase.

In intact cells, PKD can be activated by treatment with pharmacological agents, including phorbol esters, cell-permeant DAGs and bryostatin 1, or by physiological stimuli that elevate DAG levels, including neuropeptide agonists and PDGF [10,12,14–17]. Several lines of evidence indicate that PKD is activated by these agents through a novel PKC-dependent signal transduction pathway [12,14,16] that is dependent on the phosphorylation of two activation loop sites, namely Ser-744 and Ser-748 [18]. Thus, PKC inhibitors (with no direct activity towards PKD) prevent PKD phosphorylation and activation in response to phorbol esters and mitogens. Furthermore, the expression of constitutively activated mutants of the novel PKCs ε and η is sufficient to induce PKD activation [12,19,20]. Recent results demonstrating that PKCη can interact with the PH domain of PKD [19] also indicate a direct link between PKCs and PKD.

In the present study, we examined whether direct binding of phorbol esters to the CRD of PKD in vivo is necessary for PKC-dependent PKD activation. We demonstrate that exposure of intact cells to phorbol esters induces a striking CRD-dependent translocation of PKD from the cytosol to the plasma membrane, as shown by real time visualization of a functional green fluorescent protein (GFP)-PKD molecule as well as by biochemical fractionation. Using a translocation-impaired PKD mutant which contains a single amino acid substitution (P287G) in the second cysteine-rich motif, we show that phorbol ester-induced translocation of PKD is not required for PKD activation. We conclude that translocation and activation are distinct processes that operate in parallel to regulate PKD in intact cells.

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Abbreviations: CRD, cysteine-rich domain; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; PKD, protein kinase D

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2. Materials and methods

2.1. Cell culture

COS-7 cells were plated in 60 mm dishes at 3×10^5 cells/dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and were used for transfection 24 h later. Exponentially growing COS-7 cells, 40–60% confluent, were transfected with cDNA constructs using lipofectin (Life Technologies) as previously described [9,12]. The mast cell line, RBL 2H3, was maintained in DMEM supplemented with 10% fetal bovine serum. RBL 2H3 cells (1.5×10^7 cells) were transfected with cDNA constructs by electroporation (310 V, 960 μF), as previously described [21], seeded onto sterile glass coverslips and allowed to recover overnight.

2.2. cDNA constructs

PKD expression constructs containing a point mutation within the CRD (P287G) or a mutant lacking the entire CRD (ΔCRD) have previously been described [11]. Chimeric fusion proteins between GFP and PKD were generated by subcloning PKD constructs into the *Eco*RI site of a pEF-plink2-GFP_{C3} expression vector. GFP constructs were verified by restriction enzyme analysis and sequencing. Constitutively active PKCε and η constructs were generous gifts from Dr P. Parker [22].

2.3. Biochemical fractionation assays

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in isotonic buffer (25 mM Tris, pH 7.4, 250 mM sucrose, 2.5 mM MgAc, 10 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10 fg/ml aprotinin, 10 fg/ml leupeptin and 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride). Cells were lysed by disruption using a 1 ml Dounce homogenizer and nuclei/unbroken cells were pelleted by centrifugation at $800\times g$ for 1 min. Cytosolic (soluble) and membrane (pellet) fractions were prepared from the resulting homogenate by ultracentrifugation at $100\,000\times g$ for 20 min at 4°C. Equal protein samples from each fraction were extracted for 10 min in 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by gel electrophoresis, followed by transfer to Immobilon membranes at 100 V, 0.4 A, 4°C for 4 h in a buffer containing 200 mM glycine, 25 mM Tris, 0.01% SDS and 20% methanol. Membranes were blotted with either anti-PKD or anti-GFP antibodies and immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

2.4. Cell imaging

RBL 2H3 mast cells expressing GFP-PKD constructs and grown on sterile glass coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature. Nuclear staining using propidium iodide was carried out by permeabilization of cells in 0.1% Triton X-100, followed by sequential incubation with 500 ng/ml RNAse A (37°C, 10 min) and 0.1 μg/ml propidium iodide (at room temperature for 10 min). Coverslips were washed in PBS after each stage and mounted in 15 μl Gelvatol (Leica, St. Louis, MO, USA) which was allowed to set for at least 4 h. For live cell imaging, cells were incubated in phenol-red free medium (supplemented with 20 mM HEPES buffer, pH = 7.3) and placed inside a pre-warmed (37°C) chamber under an inverted confocal microscope. All confocal images were taken using a Zeiss Axiovert-100M inverted confocal laser microscope. GFP fluorescence was excited with an argon laser emitting at 488 nm and images were acquired using a 63× NA1.4 oil immersion lens and Zeiss LSM 510 software.

2.5. In vitro kinase assays

Cells were washed twice in ice-cold PBS and lysed in 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 fg/ml aprotinin, 10 fg/ml leupeptin, 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100 (lysis buffer). PKD was immunoprecipitated at 4°C for 2 h with the PA-1 antiserum (1:100 dilution), previously described [9]. Immunocomplexes were recovered with Protein A-agarose beads, washed twice in lysis buffer and once in kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM dithiothreitol). PKD autophosphorylation was determined by incubating immunocomplexes with 15 μl kinase buffer containing 100 μM [γ-^{32}P]ATP, final concentration. After incubation at 30°C for 10 min, reactions were terminated by the addition of 2× SDS-PAGE sample buffer and analyzed by gel electrophoresis and autoradiography. Exogenous substrate phosphorylation by PKD was measured by the incorporation of radioactivity into syntide-2, as previously described [6,9].

2.6. [^{3}H]Phorbol 12,13-dibutyrate (PDB) binding assays

[^{3}H]PDB binding to intact COS-7 cells was performed by incubating cells in DMEM containing 1 mg/ml bovine serum albumin and 20 nM [^{3}H]PDB at 37°C for 30 min [11]. The cells were then rapidly washed with ice-cold PBS containing 1 mg/ml bovine serum albumin and lysed with NaOH-SDS. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabelled PDB.

2.7. Materials

ECL reagents, [^{3}H]PDB and [γ^{32}P]ATP (370 MBq/ml) were from Amersham International (UK). PDB was from Sigma and the PKC inhibitors, GF 109203X and Ro 31-8220 were from LC Laboratories. Protein A-agarose was from Boehringer Mannheim. All other reagents were from standard suppliers or as indicated in the text.

3. Results

3.1. Generation of biologically active GFP-PKD chimeric fusion proteins

In order to examine PDB-induced translocation of PKD, we generated chimeric fusion proteins between GFP from *Aequorea victoria* and either the wild-type (WT) PKD (GFP-PKD) or a PKD deletion mutant lacking the tandem repeat of the cysteine-rich motifs which constitutes the DAG/phorbol ester binding domain of PKD (GFP-PKD ΔCRD) (Fig. 1A). GFP, which forms an independent 30 kDa domain with inherent fluorescence, has provided a valuable reporter molecule in the cellular localization of multiple proteins [23], including phorbol ester-regulated PKCs [24–26]. As shown in Fig. 1A, the GFP-PKD molecule, when transiently expressed in either COS-7 or RBL 2H3 cells, had the expected molecular mass (140 kDa) and could easily be distinguished from the endogenous PKD (110 kDa) of RBL 2H3 cells, as shown by Western blotting of whole cell lysates with either anti-PKD or anti-GFP antibodies. The results presented in Fig. 1A also show the expression of GFP-PKD ΔCRD.

Next, we verified that the GFP fusion protein GFP-PKD displays biological properties similar to that of native PKD, as judged by phorbol ester-mediated PKD activation and phorbol ester binding. As shown in Fig. 1B, GFP-PKD, like PKD, displayed a very low catalytic activity in resting transfected COS-7 cells but could be markedly activated by treatment of the intact cells with PDB. Similarly, COS-7 cells transfected with either PKD or GFP-PKD showed an ∼9-fold increase in specific [^{3}H]PDB binding as compared with that obtained in COS-7 cells transfected with the vector alone (Fig. 1C). No significant increase in specific [^{3}H]PDB binding was detected in COS-7 cells expressing either PKD ΔCRD or GFP-PKD ΔCRD (Fig. 1C), despite similar levels of expression of all the PKD constructs (results not shown). These results indicate that the fusion of GFP to PKD has no detectable effect on the basal catalytic activity, phorbol ester binding and kinase activation of PKD.

3.2. Phorbol ester treatment induces the translocation of GFP-PKD to the plasma membrane of RBL 2H3 cells

As these experiments demonstrated that GFP-PKD was functional, we next examined whether GFP-PKD, transiently transfected into the mast cell line RBL 2H3, undergoes trans-

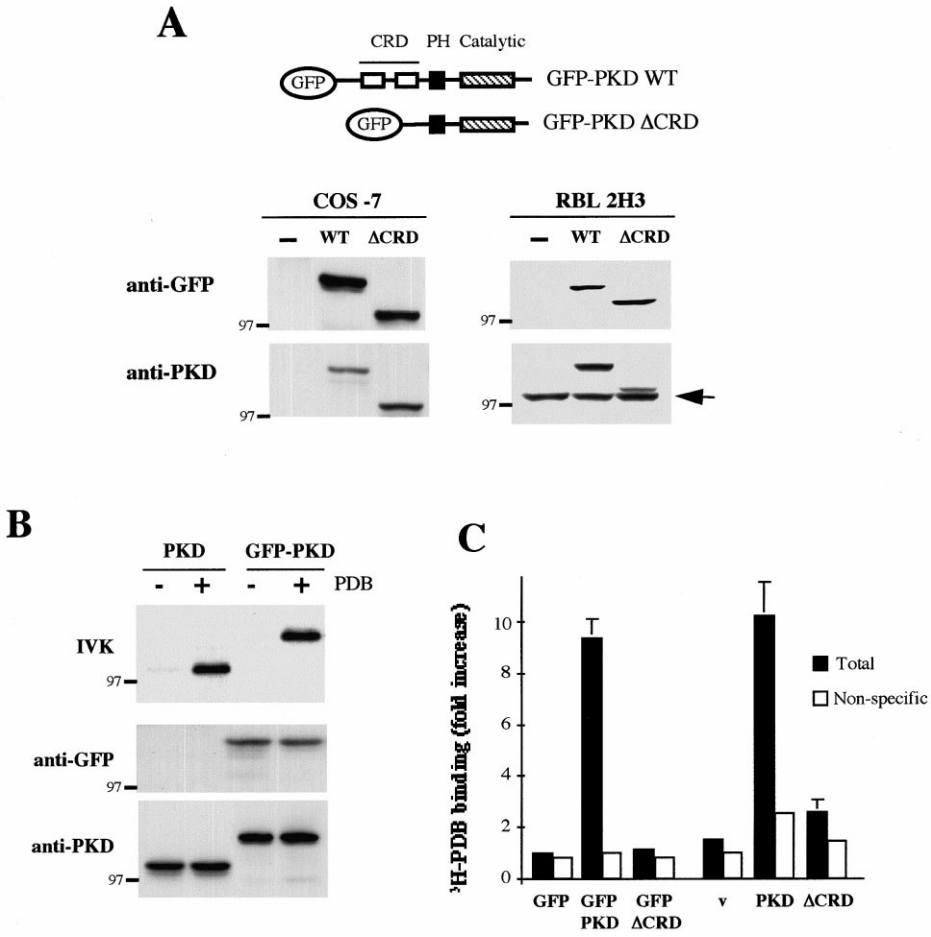

Fig. 1. Expression, activation and phorbol ester binding of GFP-PKD. (A) (upper) Schematic representation of GFP-PKD WT and a PKD deletion mutant lacking the CRD (GFP-PKD ΔCRD), fused to the C-terminus of GFP. Lower: anti-GFP and anti-PKD Western blots of COS-7 and RBL 2H3 cells transfected with the GFP-PKD constructs are shown. Endogenous PKD present in RBL 2H3 cells is indicated by an arrow. Molecular weight markers are indicated. (B) In vitro kinase assays of WT PKD and GFP-PKD expressed in COS-7 cells, either left unstimulated (−) or treated with 200 nM PDB for 15 min (+). PKD activity was measured by autophosphorylation as described under Section 2. Western blots (with anti-GFP and anti-PKD antibodies) are shown below to confirm equal expression levels of the two PKD constructs. (C) Total (black columns) and non-specific (open columns) binding of [^{3}H]PDB to COS-7 cells expressing vector alone, GFP-PKD, GFP-PKD ΔCRD, PKD or PKD ΔCRD. Data are the mean ± S.E.M. of two independent experiments, each carried out in duplicate, and are expressed as the fold increase over background.

location in response to PDB. The confocal images illustrated in Fig. 2A show that both GFP-PKD and GFP-PKD ΔCRD (shown in green) are expressed throughout the cytosol and are excluded from the nuclei (shown in red) of resting RBL 2H3 cells, as shown by the overlay of the images. Treatment with PDB resulted in a striking translocation of GFP-PKD from the cytosol to the plasma membrane (Fig. 2A, upper panels). After 15 min stimulation with PDB, 85 ± 5% of the cells ($n = 3$) show plasma membrane localization of GFP-PKD. This plasma membrane localization of GFP-PKD was maintained for at least 3 h in the continued presence of PDB (results not shown). In contrast, phorbol ester treatment had no effect on the cytosolic localization of GFP-PKD ΔCRD (Fig. 2A, lower panels). This mutant remained in the cytosol in 95 ± 1.5% ($n = 3$) of PDB-treated RBL 2H3 cells, even 1 h after treatment (results not shown).

GFP-PKD translocation in response to PDB could also be demonstrated by biochemical fractionation of transiently transfected RBL 2H3 cells, i.e. when cytosolic and membrane fractions from resting and PDB-stimulated cells were prepared. Western blotting with an anti-GFP antibody revealed that PDB induced the translocation of GFP-PKD from cytosolic to membrane fractions (Fig. 2B). Again, no phorbol ester-mediated translocation of the GFP-PKD ΔCRD mutant was detected (Fig. 2B). Thus, phorbol ester binding to the CRD of PKD induces the translocation of PKD to the plasma membrane.

3.3. PKD activation is dissociatable from PKD translocation

The two cysteine-rich motifs (cys1 and cys2) within the CRD of PKD are not functionally equivalent, with cys2 acting as the major phorbol ester binding site both in vitro and in vivo [11]. In fact, phorbol ester binding to PKD is greatly reduced by a single amino acid substitution (P287G) within the cys2 motif [11]. In the present study, we utilized this mutant (PKD P287G) to determine whether PKD translocation to the plasma membrane is required for PKC-mediated PKD activation.

Living RBL 2H3 cells, transiently transfected with GFP-PKD or GFP-PKD P287G, were imaged before and during stimulation with 200 nM PDB to visualize PKD re-distribution in real time. Translocation of GFP-PKD from the cytosol

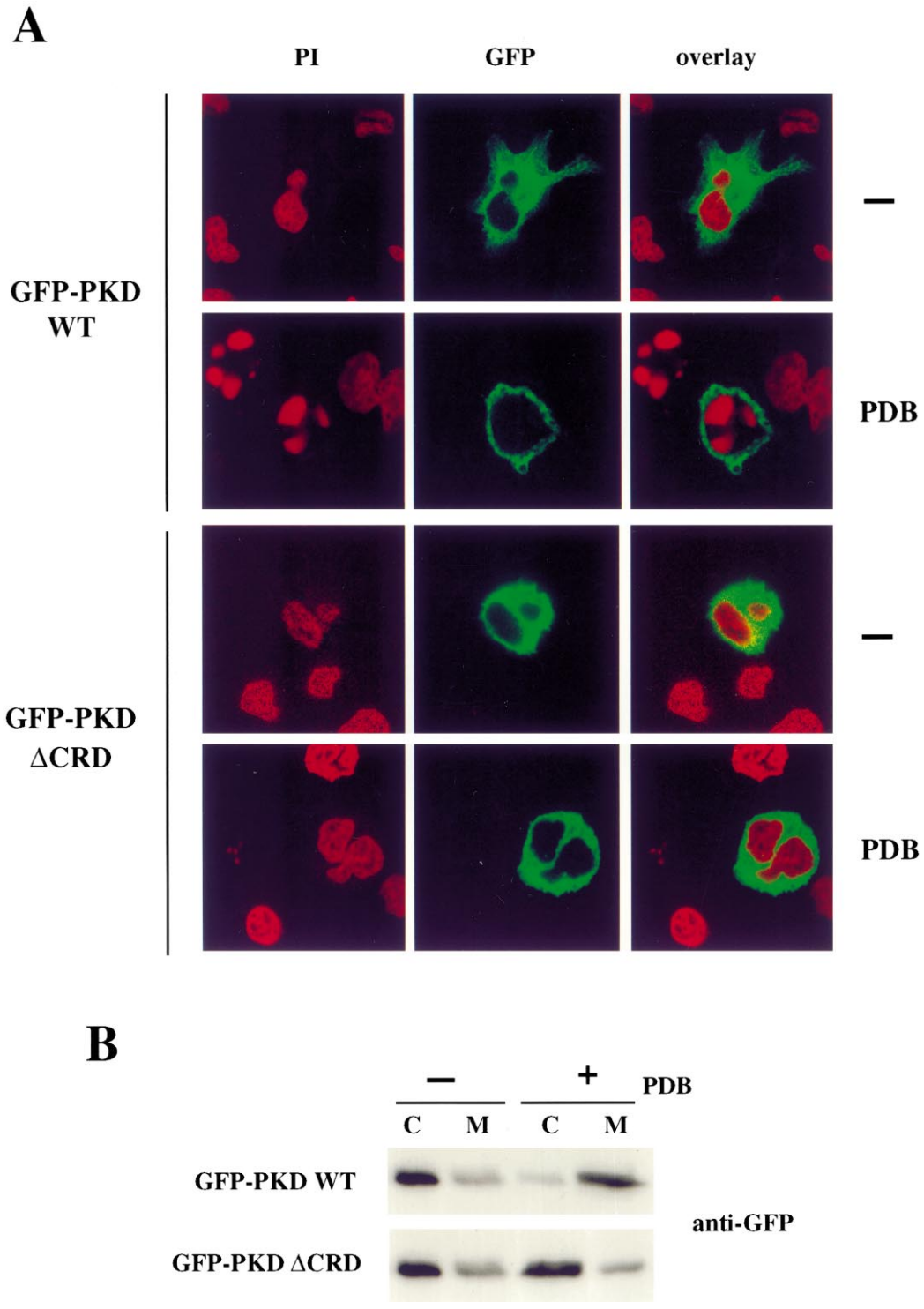

Fig. 2. Translocation of GFP-PKD and GFP-PKD ΔCRD in RBL 2H3 cells. (A) Confocal images of paraformaldehyde-fixed, transfected RBL 2H3 cells expressing GFP-PKD WT and GFP-PKD ΔCRD, stimulated with (PDB) or without (−) 200 nM PDB for 15 min. GFP is shown in green and nuclei are shown counterstained with propidium iodide (red). Results are representatives of three independent experiments. (B) Biochemical fractionation of RBL 2H3 cells expressing GFP-PKD WT and GFP-PKD ΔCRD, stimulated with (+) or without (−) 200 nM PDB for 15 min. Cytosolic (C) and membrane (M) fractions were prepared as described under Section 2 and Western-blotted with an anti-GFP antibody. Identical results were obtained in two independent experiments.

to the plasma membrane and to vesicle-like structures within the cytosol was detectable within 5 min after the addition of PDB and maximal PKD re-distribution was seen after 15 min (Fig. 3A, upper panels). In contrast, addition of PDB did not induce any significant changes in the subcellular localization of GFP-PKD P287G over the same time period (Fig. 3A, lower panels).

To determine whether PKD translocation is necessary for PKD activation, we measured the kinetics of PDB-mediated activation of both GFP-PKD and GFP-PKD P287G in RBL

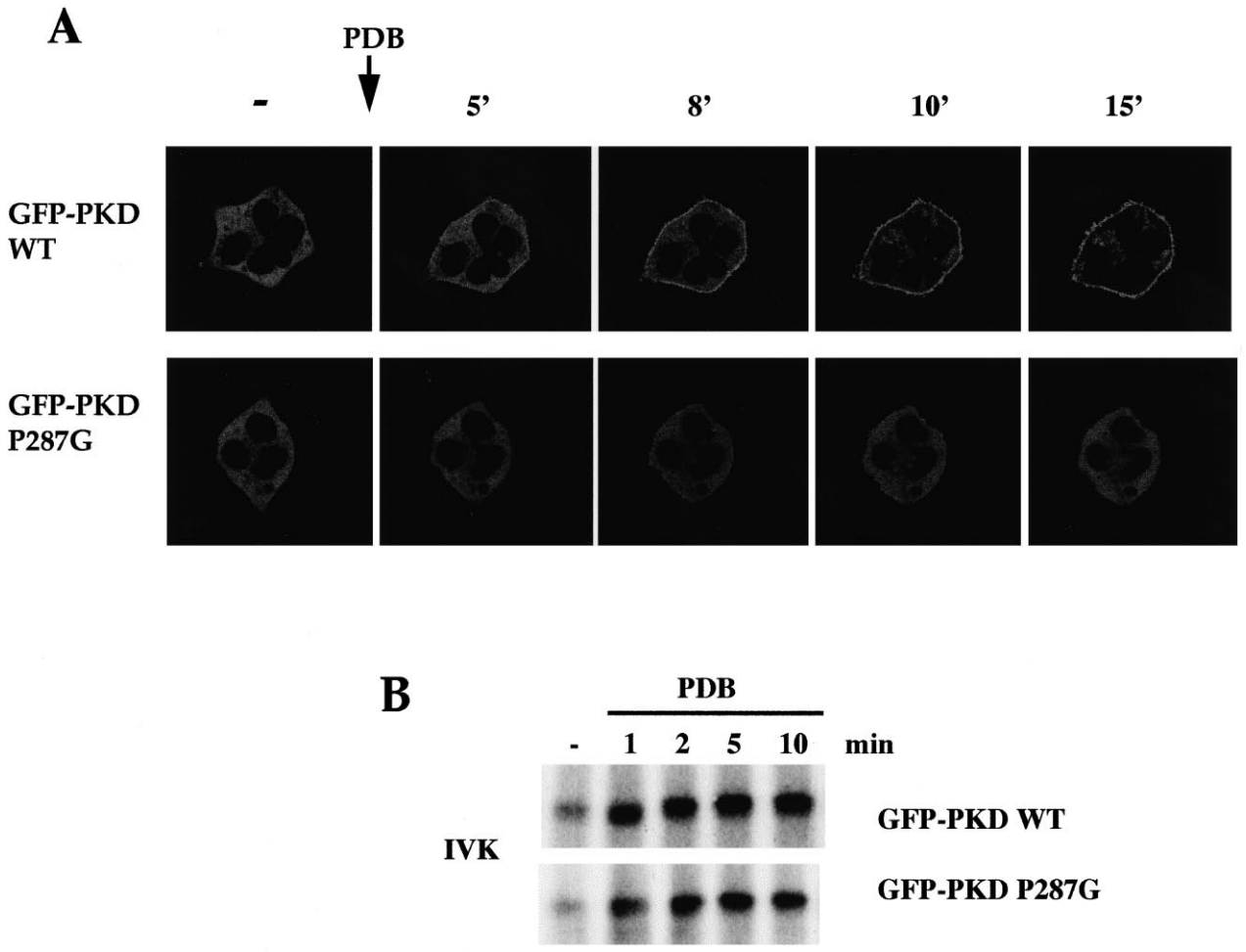

Fig. 3. Dissociation of PKD activation and translocation in RBL 2H3 cells. (A) RBL 2H3 cells, expressing GFP-PKD WT (upper) and GFP-PKD P287G (lower) were stimulated with 200 nM PDB, up to 15 min. Cells were maintained at 37°C and confocal images were taken every 30 s. Similar results were obtained in four independent experiments. (B) RBL 2H3 cells expressing GFP-PKD WT and GFP-PKD P287G were left unstimulated (−) or were treated with 200 nM PDB for increasing periods of time (1–10 min). In vitro kinase assays were performed and PKD activity was measured by autophosphorylation. Data are representatives of two independent experiments.

2H3 cells, in parallel experiments. Lysates of cells treated with 200 nM PDB for various times were immunoprecipitated with an antibody directed against the COOH-terminal region of PKD. The resulting immune complexes were incubated with [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography to determine the level of enzyme autophosphorylation. As shown in Fig. 3B, the time-courses of GFP-PKD and GFP-PKD P287G activation were identical. Interestingly, the activation of GFP-PKD, which was evident as early as within 1 min of treatment with PDB, preceded its translocation to the plasma membrane, providing further evidence that PKD activation is not dependent on PKD translocation.

3.4. PKC-dependent activation of both PKD WT and PKD P287G in intact COS-7 cells

To substantiate the conclusions obtained with the GFP-PKD chimeric proteins, we also examined PDB-induced activation and translocation of PKD and PKD P287G expressed in COS-7 cells. As illustrated in Fig. 4, the PDB dose-response curves for the activation of PKD and PKD P287G were identical, as shown both by autophosphorylation (Fig. 4A) and by exogenous substrate (syntide-2) phosphorylation (Fig. 4B). In striking contrast, the PDB dose-response curves for the translocation of WT PKD and PKD P287G (measured by biochemical fractionation) were clearly different (Fig. 4C). For example, at a concentration of 0.2 μM PDB, both WT PKD and PKD P287G were activated to the same extent and more than 95% of WT PKD had translocated from the cytosolic fraction. However, less than 10% of PKD P287G had translocated from the cytosol at this concentration of PDB. These results further substantiate that CRD-dependent translocation of PKD to the plasma membrane is not required for PKD activation.

Since the conclusion that PKC-dependent PKD activation and membrane translocation are separate events relies on the results obtained with PKD P287G, we verified that the activation of this PKD mutant in response to PDB is, in fact, mediated by PKC, as has previously been demonstrated with WT PKD. As shown in Fig. 5A, pretreatment with GF 109303X or Ro 31-8220, specific PKC inhibitors with no direct action on PKD [12,14,16], dramatically inhibited PDB-induced activation of either WT PKD or PKD P287G, both in COS-7 cells and in RBL 2H3 cells, indicating that PKD P287G like WT PKD requires functional PKC isoforms for its activation. Furthermore, co-transfection of PKD or PKD P287G together with constitutively activated PKCε* or η*

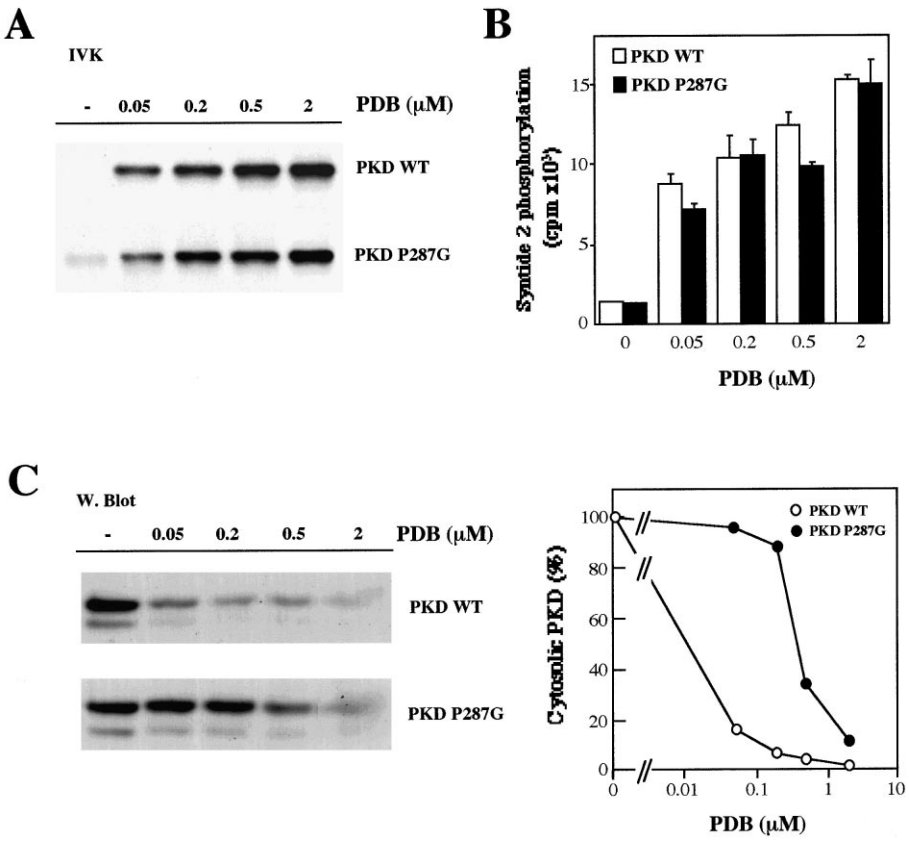

Fig. 4. PKD activation is dissociatable from its translocation in COS-7 cells. (A and B) In vitro kinase assays of PKD WT and PKD P287G expressed in COS-7 cells, after stimulation with increasing concentrations of PDB (0–2 μM) for 15 min. PKD activity was measured either by autophosphorylation (A) or by exogenous substrate (syntide-2) phosphorylation (B). (C) COS-7 cells expressing PKD WT and PKD P287G were stimulated with various concentrations of PDB (0–2 μM) for 15 min, as indicated. Cytosolic fractions were prepared as described previously and Western blotted with an anti-PKD antibody (left). Blots were quantitated by densitometry and the results expressed as the percentage of cytosolic PKD levels in control untreated cells (right). All results shown are representatives of three individual experiments.

isoforms was sufficient to induce activation of both PKD forms in RBL 2H3 cells (Fig. 5B). This activation was only slightly further increased by treatment of the intact cells with PDB. No such increases in the basal activity of WT or P287G mutant PKD were detected when they were co-transfected with vector alone, although they could be activated by treatment with PDB (Fig. 5B). Thus, activation of PKD P287G, like WT PKD, occurs through a PKC-dependent signal transduction pathway.

4. Discussion

Protein targeting to specific cellular locations as the result of increases in lipid second messengers or the activation of adaptor molecules has become a recognized mechanism for regulating complex signaling cascades [27–29]. In the present study, we used visualization of GFP-PKD chimeras to demonstrate that treatment with phorbol ester induces a striking translocation of PKD from the cytosol to the plasma membrane. PDB-induced translocation was completely prevented by deletion of the CRD and severely impaired by a single amino acid substitution in the second cysteine-rich motif of the CRD, in agreement with recent results demonstrating that this motif is responsible for mediating PDB binding to PKD [11].

Previous results demonstrated that PKD is activated by phosphorylation through a novel PKC-dependent signal transduction pathway in vivo [12,14,16]. More recently, the residues Ser-744 and Ser-748 in the activation loop of PKD have been identified as critical activating phosphorylation sites [18]. Here, we examined whether direct phorbol ester binding to PKD CRD leading to its membrane translocation is necessary for PKC-dependent activation. A salient feature of the results presented here is that PKD activation could be clearly dissociated from its membrane translocation. Specifically, a single amino acid substitution in the second cysteine-rich motif of PKD (P287G) greatly impaired PDB-induced membrane translocation but did not affect PDB-induced PKD activation. In addition, kinetic analysis revealed that phorbol ester-mediated PKD activation preceded its membrane translocation. Our results support a model in which direct binding of phorbol esters to PKD leading to its membrane translocation is not required for PKC-dependent PKD activation.

An important implication of the results presented here is that the regulation of PKD can be distinguished from that of other signal transducing protein kinases that undergo second messenger-regulated intracellular translocation. For example, Akt/PKB translocates to the plasma membrane in response to signal-dependent production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 and is only then phosphorylated and activated by two separate phosphoinositide-dependent protein kinases (reviewed in [28]). Similarly, Raf requires Ras-GTP-mediated

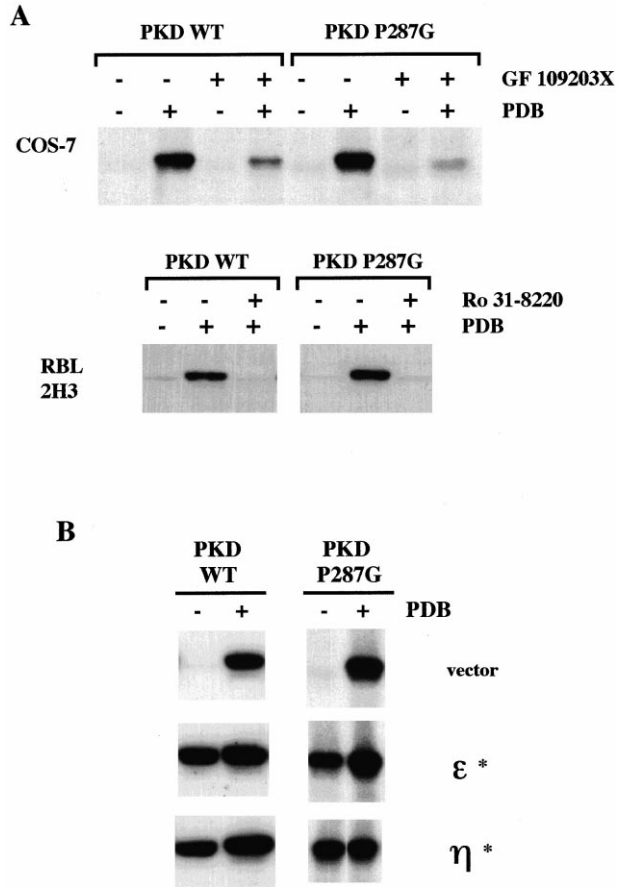

Fig. 5. PKD P287G, like PKD, is activated via PKC in vivo. (A) PKD WT and PKD P287G constructs were expressed either in COS-7 cells (upper) or in RBL 2H3 cells (lower). Cells were left untreated (−) or were pretreated (+) with the PKC-specific inhibitors, GF 109203X (3.5 μM) or Ro 31-8220 (2.5 μM), for 1 h, as indicated. Cells were subsequently left unstimulated (−) or were treated with 200 nM PDB for 15 min (+) and PKD activity was measured using in vitro kinase assays. (B) RBL 2H3 cells were co-transfected with either vector control, PKCη* or PKCε* constructs in the presence of either PKD WT or PKD P287G, as indicated. Cells were then left unstimulated (−) or were treated with 200 nM PDB for 10 min (+) and PKD activity was determined using in vitro kinase assays. Data are representatives of two independent experiments.

membrane translocation for activation [30,31]. In these cases, translocation is an obligatory step that precedes enzyme activation. In contrast to these signal-regulated protein kinases, we conclude that PKD translocation and activation are distinct processes that operate in parallel to regulate the activity and localization of this enzyme in intact cells.

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