Role of the Regulatory Domain of Protein Kinase D2 in Phorbol Ester Binding, Catalytic Activity, and Nucleocytoplasmic Shuttling

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Protein kinase D2 (PKD2) belongs to the PKD family of serine/threonine kinases that is activated by phorbol esters and G protein-coupled receptors (GPCRs). Its C-terminal regulatory domain comprises two cysteine-rich domains (C1a/C1b) followed by a pleckstrin homology (PH) domain. Here, we examined the role of the regulatory domain in PKD2 phorbol ester binding, catalytic activity, and subcellular localization: The PH domain is a negative regulator of kinase activity. C1a/C1b, in particular C1b, is required for phorbol ester binding and gastrin-stimulated PKD2 activation, but it has no inhibitory effect on the catalytic activity. Gastrin triggers nuclear accumulation of PKD2 in living AGS-B cancer cells. C1a/C1b, not the PH domain, plays a complex role in the regulation of nucleocytoplasmic shuttling: We identified a nuclear localization sequence in the linker region between C1a and C1b and a nuclear export signal in the C1a domain. In conclusion, our results define the critical components of the PKD2 regulatory domain controlling phorbol ester binding, catalytic activity, and nucleocytoplasmic shuttling and reveal marked differences to the regulatory properties of this domain in PKD1. These findings could explain functional differences between PKD isoforms and point to a functional role of PKD2 in the nucleus upon activation by GPCRs.

INTRODUCTION

The protein kinase D (PKD) family of serine/threonine kinases belongs to a subfamily of the calcium-calmodulin kinase-like superfamily (Manning *et al.*, 2002) that comprises PKD1/protein kinase C μ , PKD2 (Sturany *et al.*, 2001, 2002), and PKD3/protein kinase C ν (Hayashi *et al.*, 1999). The members of this kinase family exhibit a high degree of homology among each other particularly in their catalytic domain, which vastly differs from the classical, novel, and atypical members of the protein kinase C (PKC) family. PKDs are major targets for tumor promoting phorbol esters and are also activated via G protein-coupled receptors (GPCRs) and activation of PKC. PKD1 is the best characterized isoform of this family so far and has been implicated in various biological processes, including the regulation of Golgi function, cell proliferation, and apoptosis (Rykx *et al.*, 2003).

The various biological roles of PKD1 are regulated by both its catalytic activity and its subcellular localization that are modulated by its regulatory domain (Iglesias and Rozengurt, 1999; Oancea *et al.*, 2003). Functional differences between PKD isoforms could therefore arise from distinct ef-

fects of the regulatory domain on the catalytic activity and the subcellular localization of the respective enzyme.

The regulatory domain at the NH₂-terminal region of PKDs consists of a tandem repeat of cysteine-rich zinc finger-like motifs termed C1a and C1b and a pleckstrin homology (PH) domain (Rykx *et al.*, 2003). Cysteine-rich zinc finger-like motifs are present in a variety of proteins, including PKCs (Wang *et al.*, 2003), chimaerin (Areces *et al.*, 1994), and UNC-13 (Kazanietz *et al.*, 1995), and they play multiple roles in enzyme regulation. In PKD1, the C1a/C1b domain is responsible for the majority of phorbol ester binding (Iglesias *et al.*, 1998) and acts as a membrane targeting module that shuttles PKD1 between different subcellular compartments (Rykx *et al.*, 2003). Furthermore, deletion of the C1a, the C1b, or the PH subdomain, respectively, leads to the constitutive activation of PKD1, suggesting that these subdomains have a negative/inhibitory impact on its catalytic activity (Iglesias and Rozengurt, 1998). The PH domain also has been shown to mediate nuclear export, whereas the C1b domain regulates nuclear import of PKD1 (Rey *et al.*, 2001). At present little is known about whether the various PKD isoforms are regulated and act in a similar manner or exhibit distinct regulatory and functional properties. There is controversy as to the localization of PKD2. It has been suggested that in contrast to PKD1 and 3, PKD2 activation does not induce its redistribution from the cytoplasm to the nucleus (Rey *et al.*, 2003a). Furthermore, we have recently demonstrated that in contrast to PKD1, activation of nuclear factor- κ B by PKD2 in response to oxidative stress does not require its catalytic activity (Mihailovic *et al.*, 2004). Thus, PKD2 could have distinct regulatory properties compared with the other PKD isoenzymes.

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Here, we have analyzed the role of the regulatory domain of PKD2 in phorbol ester binding, the control of catalytic activity, and its subcellular localization by using live cell imaging. We show that the PH domain in PKD2 is a negative regulator of enzyme activity, which is similar to PKD1. However, in marked contrast to PKD1, the C1a/C1b domain of PKD2 plays a positive/stimulatory role in the regulation of the catalytic activity. In particular the C1b motif is required for phorbol ester binding as well as basal and GPCR-stimulated kinase activity of PKD2. Using live cell imaging of PKD2 fused to green fluorescent protein (EGFP), we show that PKD2 shuttles continuously between the cytoplasm and the nucleus of epithelial tumor cells. Activation of the cholecystokinin (CCK)_B receptor provokes nuclear accumulation of PKD2. Mutational analysis of the regulatory domain revealed a functional nuclear export signal (NES) in the C1a domain and a functional nuclear localization sequence (NLS) in the linker region between the C1a and the C1b domain of PKD2. Thus, there is a sophisticated regulation of PKD2 activity and nucleocytoplasmic shuttling by the cysteine-rich domain of PKD2. The C1b domain is mainly responsible for phorbol-12,13-dibutyrate (PDBu) binding and catalytic activity, whereas the C1 domain and the linker region regulate nucleocytoplasmic shuttling. These data point to a functional role of PKD2 in the nucleus and demonstrate marked differences in the control of catalytic activity and subcellular localization of PKD1 and PKD2 by their regulatory domain, which could determine isoform-specific signaling properties.

MATERIALS AND METHODS

Cell Culture

Stock cultures of human embryonic kidney (HEK)293, AGS-B, and Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO₂ at 37°C. AGS-B cells are AGS human gastric cancer cells (American Type Culture Collection, Manassas, VA) stably transfected with the expression construct CCK_B-pcDNA1-neo comprising the full-length coding region of the human CCK_B/gastrin receptor and the neomycin resistance gene (Sturany *et al.*, 2002).

Generation of PKD2 Mutants

PKD2 and PKD2 mutants were ligated into pEGFP-C2 (BD Biosciences Clontech, Palo Alto, CA) or a pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA) containing a N-terminal FLAG-tag (a kind gift of Dr. Franz Oswald, University of Ulm, Ulm, Germany). Deletion of the entire C1a/C1b domain (H139-C314; 176 amino acids) and deletion of the PH domain (T397-M509; 113 amino acids) were performed directly in pcDNA3 by a splice-overlap PCR strategy using *Taq*-Polymerase (Invitrogen). These primers were used resulting in the deletion of the C1a/C1b domain: EcoRI5' (5' gcgaattcgccaccgcccctctt 3'), 3'ΔC1a/C1b (5' ctcccaccgcccggcgatctggaa 3'), 5'ΔC1a/C1b (5' atccgcccgtggggaggccctt 3'), and 3'XbaI (5' gaatggcgccctctagatcatgc 3'). These primers resulted in the deletion of the PH domain: EcoRI5' (5' gcgaattcgccaccgcccctctt 3'), 3'ΔPH (5' gatgacggggctgattccgcgt 3'), 5'ΔPH (5' aaatccagcccgtatctcttcag 3'), and 3'XbaI (5' gaatggcgccctctagatcatgc 3'). Deletions of the C1a domain (H139-P185; 47 amino acids) and C1b domain (H265-C314; 50 amino acids) were made in pEGFP-PKD2 by PCR with primers containing specific restriction sites. The deletion of the C1a domain was established with primers EcoRI5' (5' gcgaattcgccaccgcccctctt 3'), 3'ApaI (5' ccgtgaggcccccggggcg 3') and cloned into EGFP-PKD2. Primers for the deletion of the C1b domain are the following: EcoRI5' (5' gcgaattcgccaccgcccctctt 3'), 3'Sall (5' cgacgctgcaccttgagagc 3'), 5'Sall (5' acgctgcactgctctggggag 3'), and 3'XbaI (5' gaatggcgccctctagatcatgc 3'). Site-specific mutations within EGFP-PKD2, resulting both in single and double amino acid substitutions (D⁶⁹⁵A, S⁷⁰⁶/V⁷¹⁰E, L¹⁵⁹/V¹⁶³A, and K¹⁹³A/R¹⁹⁴G) were performed by a PCR approach using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All these constructs were confirmed by DNA sequence analysis.

AGS-B, HEK293, and PANC-1 Cell Transfection

Exponentially growing AGS-B, HEK293, or PANC-1 cells (5 × 10⁴ cells/35 mm dish) were transfected with EGFP- or FLAG-tagged PKD2 or the respective PKD2 mutants as indicated in the figure legends using FuGENE (Roche

Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The cells were used for experimental purposes 48 h after transfection.

PDBu Binding to HEK293 Cells

To determine the binding of [³H]PDBu to intact HEK293 cells, cells transfected with either pEGFP, pEGFP-PKD2, or the respective mutants were washed twice with DMEM and incubated with binding medium (DMEM containing 1 mg/ml bovine serum albumin and 10 nM [³H]PDBu) at 37°C for 30 min. Cells were then rapidly washed at 4°C with phosphate-buffered saline (PBS), lysed, and bound radioactivity was determined using a Beckman beta-scintillation counter. Nonspecific binding was determined in the presence of 10 μM PDBu.

Western Blotting and Immunoprecipitations

For immunoprecipitations, HEK293 or AGS-B cells in 35-mm dishes were transfected with various plasmids as described above and lysed in a solution containing 50 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol (DTT), protease inhibitors (aprotinin [10 μg/ml], leupeptin [100 μg/ml], pepstatin [0.7 μg/ml], 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride), and 1% Triton X-100 (lysis buffer I). Proteins were immunoprecipitated with the anti-EGFP or anti-FLAG antibody and further analyzed by Western blotting using various antibodies as indicated in the figure legends. Immunoreactive bands were visualized using horseradish peroxidase-conjugated antihorse IgG followed by enhanced chemiluminescence (ECL).

Crm-1 Binding Assay

Nuclear extracts of HEK293 cells transfected with FLAG-PKD2 constructs were prepared from 5 × 10⁶ cells as described by Dignam *et al.* (1983). Immunoprecipitations were performed for 1 h using an anti-FLAG monoclonal antibody (mAb) (Sigma, St. Louis, MO), respectively. Western Blots were performed as described above.

In Vitro Kinase Assays

To examine PKD2 autokinase activity and in vitro histone phosphorylation by PKD2 and its mutants, anti-EGFP immunoprecipitates were prepared as described above. Immune complexes were washed twice with lysis buffer I, twice with lysis buffer II (buffer I without Triton X-100) and twice with kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 1 mM DTT) and then resuspended in 20 μl of kinase buffer in the presence (for histone phosphorylation) or absence (for autokinase activity) of 0.5 mg/ml histone H1 and 100 μM [^γ-³²P]ATP. Reactions were incubated for 10 min at 30°C, terminated by adding an equal amount of 2× SDS-PAGE sample buffer, and further analyzed by SDS-PAGE, followed by autoradiography. Autoradiographs were further analyzed by scanning densitometry using the MacBAS V2.5 software (Fuji Photo Film, Tokyo, Japan).

Immunofluorescence Studies

After the indicated transfections and stimulations, AGS-B cells were immediately fixed with 4% formaldehyde for 10 min. Antibodies were added overnight in PBS at 4°C. After washing in PBS, cells were incubated with Alexa-coupled secondary antibodies (Dianova, Hamburg, Germany) for 1 h at room temperature. Slides were finally embedded in Mowiol (Calbiochem, San Diego, CA).

Live Cell Imaging and Fluorescence Intensity Evaluation

Imaging of living and fixed cells expressing EGFP-tagged proteins was performed with a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany) equipped with a 488-nm argon laser. For live cell imaging cells were grown and transfected in MatTek glass-bottomed dishes (MatTek, Ashland, MA). Forty-eight hours after transfection cells were maintained in DMEM supplemented with 30 mM HEPES, pH 7.0. During the measurements the medium was kept at 37°C in an atmosphere containing 5% CO₂ using a LSM 510 Incubator S (Carl Zeiss). Gastrin (100 nM) stimulation of the cells was performed 40 min before imaging or fixation, respectively. After incubation with LMB cells were maintained at 37°C for 1 h before imaging or fixation. Samples were imaged with detector slit widths of 500–548 nm using a 63× oil (1.4 numerical aperture) immersion objective. Quantitative analysis of the nuclear and cytoplasmic fluorescence intensity was performed on images of the midsection of living or fixed cells. The midsection was first determined by a Z-stack. The nuclear/cytoplasmic ratio of fluorescence intensity was quantified using the Image J public domain Java image processing program (<http://rsb.info.nih.gov/ij/download.htm>). For quantification, the fluorescence intensity in the cytoplasmic or nuclear compartment was determined in a 0.5 × 0.5-μm square that was centered in the nucleus or cytoplasm, respectively (2 values per cell). Due to the more inhomogeneous cytoplasmic distribution of EGFP-PKD2 in gastrin stimulated AGS-B cells four values in different areas of the cytoplasm were examined and combined to generate the cytoplasmic value. Results are the means of the fluorescence intensity determined in 20 cells expressing EGFP-PKD2-WT or the respective

EGFP-PKD2 mutants. The relative nuclear fluorescence intensity was calculated using the following equation: $F_i^{\text{nuc}}/\sum F_i^{\text{nuc}}/n/\sum F_i^{\text{cyt}}/n$. F_i^{nuc} is the fluorescence intensity in the nucleus, F_i^{cyt} is the relative fluorescence intensity in the cytoplasm, and n is the number of cells examined.

Fluorescence Loss in Photobleaching (FLIP)

To monitor nucleocytoplasmic shuttling of the EGFP fusion proteins, live cell imaging was performed as described above. A time series of images was recorded after 20-min stimulation of cells with 100 nM gastrin. The bleach rate for each series of images was calculated and used to correct the fluorescence intensities of the images. For qualitative FLIP analysis, cells imaged using the 488-nm line of an argon laser of a Zeiss LSM510 META confocal microscope operating at 40% laser power and 5% transmission (imaging intensity). Four imaging scans of a single cell were performed. Then, the cytoplasm was chosen as region of interest by the LSM 510 Meta software and selectively bleached four times with 100% transmission (bleaching intensity) 30 s each, followed by imaging scans at the times indicated in the figure. At least 10 data sets were analyzed for each time point. The background corrected nuclear fluorescence at the time before the initial photobleaching was set as 100%. The data points were fitted with a nonlinear curve using GraphPad Prism software (GraphPad Software, San Diego, CA).

Materials

[γ - 32 P]ATP (5000 Ci/mmol; 37 GBq = 1 mCi) was from Amersham Biosciences (Piscataway, NJ). [3 H]PDBu (20 Ci/mmol) was purchased from MP Biomedicals (Strasbourg, France). The anti-Golgin 97 antibody directed against a peripheral membrane protein localized on the cytoplasmic face of the Golgi apparatus was from Molecular Probes (Leiden, The Netherlands). The anti-CRM-1 and the anti-importin α /Rch-1 antibodies were from BD Transduction Laboratories (Lexington, KY). The monoclonal anti-RNA polymerase II (Pol-II) antibody was from BAbCO (Richmond, CA). The monoclonal anti-green fluorescent protein (GFP) antibody was obtained from Roche Diagnostics. The polyclonal antiphospho-PKD1/protein kinase C μ Ser⁷⁴⁴/Ser⁷⁴⁸ antibody was obtained from Cell Signaling Technology (Beverly, MA). This antibody was raised against a synthetic phospho-Ser⁷⁴⁴/Ser⁷⁴⁸ peptide corresponding to residues surrounding Ser⁷⁴⁴/Ser⁷⁴⁸. This sequence is identical in PKD1 and PKD2 with the corresponding serine residues in PKD2 being Ser⁷⁰⁶ and Ser⁷¹⁰. The anti-PKD2 antibody was from Orbigen (San Diego, CA). The anti-PKD1 antibody D-20 was purchased from Upstate Biotechnology (Charlottesville, VA). All other reagents were of the highest grade available.

RESULTS

Role of the Regulatory Domain for [3 H]PDBu Binding to PKD2

We have recently demonstrated that expression of PKD2 in HEK293 cells confers increased phorbol ester binding (Sturany *et al.*, 2001). To determine the role of the regulatory domain of PKD2 comprising the duplex cysteine-rich domains and the PH domain for phorbol ester binding, we examined EGFP-tagged mutants lacking the PH domain (PKD2- Δ PH), the entire cysteine-rich domain (PKD2- Δ C1a/C1b), or only the C1a (PKD2- Δ C1a) and C1b (PKD2- Δ C1b) domain, respectively (Figure 1). All mutants exhibited a similar level of expression upon transfection (Figure 2A). [3 H]PDBu binding of PKD2- Δ PH was comparable with wild-type PKD2. In contrast, deletion of the entire C1a/C1b domain abolished phorbol ester binding. The PKD2 mutant lacking the C1a domain was still able to bind phorbol esters, but binding was reduced by ~50% compared with wild-type PKD2 (Figure 2A). In contrast, the mutant lacking the C1b domain exhibited virtually no detectable [3 H]PDBu binding. Thus, the cysteine-rich domain and in particular the C1b domain is essential for [3 H]PDBu binding to the enzyme.

Regulation of PKD2 Kinase Activity by the Regulatory Domain

To determine the role of the C1a/C1b domain and the PH domain for PKD2 kinase activity, HEK293 cells were transfected with various EGFP-tagged PKD2 expression plasmids: wild-type PKD2; PKD2-S^{706/710}E, in which Ser⁷⁰⁶ and Ser⁷¹⁰ within the activation loop of the kinase are replaced

Figure 1. Schematic representation of the PKD2 mutants used in this study. The C1a/C1b domain is located at the amino-terminal region of wild-type PKD2 (AA H¹³⁹–C³¹⁴). The PH domain (AA T³⁹⁷–M⁵⁰⁹) is interposed between the C1a/C1b domain and the kinase domain (KD), which is located at the carboxy-terminal end. PKD2-S^{706/710}E carries a Ser to Glu mutation at positions 706 and 710 within the activation loop of the kinase. PKD2-D⁶⁹⁵A carries an Asp to Ala mutation at position 695 within the DFG motif of the kinase. PKD2- Δ C1a/C1b and PKD2- Δ PH are mutants in which the entire cysteine-rich domain (H¹³⁹–C³¹⁴) or the entire PH domain (T³⁹⁷–M⁵⁰⁹) are deleted. PKD2- Δ C1a and PKD2- Δ C1b lack the C1a domain (AA H¹³⁹–P¹⁸⁵) or the C1b domain (AA H²⁶⁵–N³¹⁵), respectively.

with Glu, rendering the kinase constitutively active (Mihailovic *et al.*, 2004); PKD2- Δ PH, a mutant lacking the PH domain; PKD2-D⁶⁹⁵A, a catalytically inactive mutant of PKD2 that was generated by replacing the Asp in the DFG motif by Ala and PKD2- Δ C1a/C1b, which lacks the entire C1a/C1b domain. All plasmids exhibited similar levels of expression (Figure 2B).

Deletion of the PH domain in PKD2 results in a marked increase in PKD2 catalytic activity as demonstrated by autokinase activity as well as in vitro kinase assays using histone as substrate. The catalytic activity of PKD2- Δ PH was comparable to the constitutively active PKD2-S^{706/710}E mutant (Figure 2B). In contrast, deletion of the C1a/C1b domain results in only a slight increase in the basal catalytic activity compared with wild-type PKD2. Thus, the PH domain is a negative regulator of PKD2 catalytic activity, whereas the C1a/C1b domain seems to have only a weak, if at all, inhibitory effect on kinase activity.

To further define the regulatory properties of the C1a/C1b domain, we examined the catalytic activity of mutants lacking the C1a or the C1b domain, respectively, in an epithelial tumor cell model, AGS-B human gastric cancer cells. Again, basal activity of PKD2- Δ C1a/C1b was only slightly increased compared with wild-type PKD2 and could be further enhanced by incubation of cells with gastrin. PKD2- Δ C1a exhibited only a small, not significant increase in basal kinase activity, which was comparable to PKD2- Δ C1a/C1b. Incubation of cells with gastrin led to a marked increase in catalytic activity of PKD2- Δ C1a, which was comparable to that of PKD2- Δ C1a/C1b and reached ~60% of wild-type PKD2 (Figure 3A). In marked contrast, PKD2- Δ C1b exhibited a rather low basal catalytic activity. Treatment of cells with gastrin led only to a small increase in catalytic activity which was comparable to that of wild-type PKD2 in unstimulated AGS-B cells. Similar data were ob-

Figure 2. (A) Role of the regulatory domain of PKD2 in [³H]PDBu binding. Top, HEK293 cells transfected with wild-type EGFP-PKD2 (WT), EGFP-PKD2-ΔPH (ΔPH), EGFP-PKD2-ΔC1a/1b (ΔC1a/C1b), EGFP-PKD2-ΔC1a (ΔC1a), and EGFP-PKD2-ΔC1b (ΔC1b) as indicated were incubated with 400 nM PDBu (+) or solvent (−) for 15 min and subsequently subjected to Western blot analysis using an anti-GFP mAb. Bottom, HEK293 cells were transfected with the EGFP vector alone, wild-type PKD2 (WT), or the PKD2 mutants as indicated and incubated with [³H]PDBu in the presence (PDBu, +) or absence (PDBu, −) of 10 μM unlabeled PDBu. [³H]PDBu binding was determined as described in *Materials and Methods*. Data are expressed as -fold increase in PDBu binding above control values, which were obtained in HEK293 cells transfected with pEGFP and are the means ± SE of at least three independent experiments. (B) Effect of the regulatory domain on PKD2 catalytic activity. HEK293 cells were transfected with wild-type PKD2 (WT) or the respective EGFP-tagged PKD2 mutants as indicated (S/E, PKD2-S^{706/710}E; ΔPH, PKD2-ΔPH; D/A, PKD2-D⁶⁹⁵A; ΔC1a/C1b, PKD2-ΔC1a/C1b). Forty-eight hours after transfection, cells were lysed, and lysates were subjected to immunoprecipitation using an anti-GFP mAb. An aliquot of the lysates was analyzed by Western blotting with the anti-GFP mAb to determine the expression of the various mutants (GFP, top). Autokinase activity (AK, left), or histone phosphorylation (His, right) were analyzed by in vitro kinase assays followed by SDS-PAGE and autoradiography. The positions of EGFP-PKD2 autokinase (AK) and histone are indicated by arrowheads (▶). PKD2 autophosphorylation and histone phosphorylation were quantified by scanning densitometry. Data shown are expressed as -fold increase in PKD2 or histone phosphorylation above wild-type PKD2 activity in unstimulated cells and are the means ± SE of at least three independent experiments.

tained when cells were incubated with PDBu (our unpublished data). Thus, the C1b domain seems to be crucial for PKD2 catalytic activity. PKD2-ΔC1b does not bind phorbol esters (Figure 2A). However, this mutant is still phosphorylated at the critical residues Ser⁷⁰⁶/Ser⁷¹⁰ in the activation loop in response to gastrin (Sturany *et al.*, 2002; Figure 3B)

Figure 3. Effect of the C1a/C1b domain on PKD2 catalytic activity in AGS-B cells. A: AGS-B cells were transfected with EGFP-PKD2 (WT), EGFP-PKD2-ΔC1a/C1b (ΔC1a/C1b), EGFP-PKD2-ΔC1a (ΔC1a), or EGFP-PKD2-ΔC1b (ΔC1b). Forty-eight hours after transfection, cells were incubated with 100 nM gastrin for 15 min as indicated. Cells were subsequently lysed and lysates subjected to immunoprecipitation with an anti-GFP mAb. Aliquots of the immunoprecipitates were analyzed by Western blotting using the anti-GFP mAb to determine the expression level of the various mutants (GFP; ▶). Immunoprecipitates were further subjected to in vitro kinase assays as described in *Materials and Methods*. Histone phosphorylation was quantified by scanning densitometry. Data shown are expressed as -fold increase in histone phosphorylation above wild-type PKD2 activity in unstimulated cells and are the means ± SE of at least three independent experiments (His; ▶). (B) Activation loop phosphorylation of PKD2, PKD2-ΔC1a, and PKD2-ΔC1b in response to gastrin. AGS-B cells were transfected with EGFP-PKD2 (WT), EGFP-PKD2-ΔC1a (ΔC1a), or EGFP-PKD2-ΔC1b (ΔC1b) and subsequently incubated with gastrin as described above. Cell lysates were subjected to immunoprecipitation with an anti-GFP mAb. Immunoprecipitates were further analyzed by Western blotting using an antibody that detects phosphorylation of PKD2 at Ser⁷⁰⁶ and Ser⁷¹⁰ (pS^{706/710}; ▶, top) and an anti-GFP mAb (GFP; ▶, bottom), respectively.

and also in response to PDBu (our unpublished data). Thus, phosphorylation of PKD2 by PKCs at these residues is not sufficient to confer maximum PKD2 catalytic activity in the absence of lipid binding to the C1b domain.

In conclusion, the C1a and the C1b motif differ substantially with respect to their effect on the catalytic activity of PKD2. The PKD2-ΔC1a/C1b mutant and in particular the less extensive deletions of the C1a and the C1b domain revealed that neither of these domains plays a negative role in the regulation of PKD2 activity. In contrast, the C1b domain seems to be required for the activation of PKD2 and can therefore be regarded as a positive regulator of PKD2 catalytic activity.

Subcellular Localization of PKD2 in AGS-B Human Gastric Cancer Cells

Regulatory domains not only modify the catalytic activity but also regulate the subcellular localization of protein kinases (Filhol *et al.*, 2003). So far, very little is known about the subcellular localization of PKD2. It has been questioned whether PKD2 can localize to the nuclear compartment under physiological conditions (Rey *et al.*, 2003a). To visualize the subcellular localization of PKD2, AGS-B human gastric cancer cells were transfected with EGFP-tagged wild-type PKD2. The majority of the EGFP signal was detected in the cytoplasm. Some fluorescence was also visible in the perinuclear region. Costaining with the Golgi marker Golgin revealed that perinuclear PKD2 largely localized to the Golgi compartment in accordance with previous data obtained in Madin-Darby canine kidney cells (Yeaman *et al.*, 2004). To further determine the subcellular localization of PKD2 in response to a physiological stimulus, AGS-B cells were treated with gastrin that potently induces activation of the kinase via the CCK_B receptor (Sturany *et al.*, 2002). Gastrin treatment of AGS-B cells provoked an accumulation of EGFP-PKD2 at the plasma membrane and in particular in the nucleus. A marked, 8.5-fold increase in the nucleo/cytoplasmic fluorescence ratio of EGFP-PKD2 could be observed (Figure 4A, top). Nuclear localization of PKD2 was confirmed by costaining of cells with an anti-Pol-II antibody that detects nuclear RNA polymerase II. In the presence of gastrin, EGFP-PKD2 and Pol-II immunoreactivity were both detectable in the nuclear compartment (Figure 4B). Endogenous PKD2 immunoreactivity was mainly detectable in the cytoplasm and the perinuclear area of unstimulated AGS-B cells (Figure 4C). We confirmed that the antibody used to detect endogenous PKD2 only detects PKD2 and no other isoforms of the PKD family (Figure 4C). On treatment of cells with gastrin, there was a 3.5-fold increase in the nucleo/cytoplasmic fluorescence ratio of endogenous PKD2 using an antibody that selectively detects the kinase. Thus, endogenous as well as transfected PKD2 accumulate in the nucleus upon incubation of AGS-B cells with gastrin.

Nucleocytoplasmic Shuttling of PKD2 Is Regulated by a Crm-1-dependent Mechanism

Next, we examined how PKD2 shuttles between the cytoplasm and the nucleus. For nuclear export, most proteins rely on a hydrophobic NES and its recognition by Crm-1, an NES receptor in the nuclear pore complex. We found that PKD2 coimmunoprecipitates with Crm-1 (Figure 5A), suggesting a Crm-1 dependent nuclear export mechanism for PKD2. The interaction of Crm-1 with PKD2 was independent of its catalytic activity (our unpublished data). Leptomycin B (LMB) is an antifungal antibiotic that has been shown to inhibit NES-dependent nuclear export by binding to Crm-1 (Kudo *et al.*, 1998; Nishi *et al.*, 1994). Indeed, LMB treatment of live AGS-B cells resulted in a striking, 24-fold increase in the nucleo/cytoplasmic fluorescence ratio of EGFP-PKD2, which was not further enhanced in the presence of gastrin (Figure 5B). Nuclear fluorescence intensity did not increase when cells were transfected with the EGFP vector alone and subsequently incubated with LMB (our unpublished data). There was also a sevenfold increase in the nucleo/cytoplasmic fluorescence ratio of endogenous PKD2 in the presence of LMB in AGS-B cells. Again, gastrin did not further increase nuclear PKD2 immunoreactivity in LMB-treated cells (Figure 5C). Thus, PKD2 is not restricted in its mobility in living cells and shuttles between the nucleus and the cytoplasm by a Crm-1 dependent mechanism.

Figure 4. Subcellular localization of PKD2 in AGS-B human gastric cancer cells. (A) AGS-B cells were transfected with EGFP-PKD2 as described in *Materials and Methods* and subsequently incubated with 100 nM gastrin (G) for 40 min as indicated. Cells were subsequently fixed and stained with an anti-Golgin (Gol) antibody to label the Golgi compartment before further analysis by confocal microscopy. The overlay of EGFP-PKD2 (green) and Golgin staining (red) is indicated by yellow. The ratio of nuclear and cytoplasmic EGFP-fluorescence intensity in the presence (G) and absence (–) of gastrin (F_{nuclear}) was quantified as described in *Materials and Methods*. White arrows indicate PKD2 at the plasma membrane. Bars, 5 μm . (B) Costaining of EGFP-PKD2 and nuclear RNA Pol-II. AGS-B cells were transfected with EGFP-PKD2 as described in *Materials and Methods* and were left untreated (top, –) or incubated with 100 nM gastrin (G) for 40 min. Cells were subsequently fixed and stained with an anti-Pol-II antibody followed by a labeling with an Alexa 568 antibody before further analysis by confocal microscopy. The overlay of EGFP-PKD2 (green) and Pol-II staining (red) is indicated by yellow. Bars, 5 μm . (C) Subcellular localization of endogenous PKD2 in AGS-B cells. Left, AGS-B cells were left untreated (–) or incubated with 100 nM gastrin (G) for 40 min, fixed, and subsequently incubated with an anti-PKD2 antibody followed by Alexa 488 staining. Images were taken as described in *Materials and Methods*. The ratio of nuclear and cytoplasmic PKD2 immunoreactivity in the presence (G) and absence (–) of gastrin (F_{nuclear}) was quantified as described in *Materials and Methods*. Right, HEK293 cells were transfected with vector controls (top two lanes) or PKD2 and PKD1, respectively (bottom two lanes). Cells were lysed, the membrane was cut after transfer, single lanes were subjected to Western blotting using a selective anti-PKD2 antibody or an anti-PKD1 antibody as indicated, and the membrane was reconstituted before ECL. The positions of PKD2 and PKD1 are indicated by arrowheads.

Figure 5. Nucleocytoplasmic shuttling of PKD2 is regulated by a Crm-1-dependent mechanism. (A) HEK293 cells were transfected with FLAG-tagged wild-type PKD2 (Flag). Forty-eight hours after transfection, nuclear extracts were prepared and subjected to immunoprecipitation with an anti-FLAG or an anti-CRM-1 antibody (IP) followed by Western blotting (WB) with an anti-Crm-1 or an anti-FLAG antibody, respectively. (B) AGS-B cells were transfected with EGFP-PKD2 and plated in MatTek glass-bottomed dishes for 48 h. Then, cells were treated with 10 ng/ml LMB for 1 h or an equivalent amount of solvent. Finally cells were incubated with 100 nM gastrin (G) for another 40 min at 37°C or received an equivalent amount of solvent (–). Representative images were captured *in vivo* and the relative nuclear fluorescence intensity was (F_{nuclear}) calculated as described in *Materials and Methods*. (C) AGS-B cells were incubated with 10 ng/ml LMB for 1 h and subsequently with 100 nM gastrin (G) for 40 min as indicated. Control cells received an equivalent amount of solvent (–). Cells were fixed and subsequently incubated with an anti-PKD2 antibody followed by indirect immunofluorescence. Representative images were captured and relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*. (D) Panc-1 cells were plated in MatTek glass-bottomed dishes and transfected with EGFP-PKD2. Forty-eight hours after transfection, cells were incubated with 10 ng/ml LMB for 1 h or solvent (–). Representative images were captured *in vivo* and the relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*. Bars, 5 μm .

The fact that very little endogenous PKD2 as well as EGFP-PKD2 is detectable in the cytoplasm upon treatment of cells with LMB suggests that most of the cellular PKD2 participates in the nucleocytoplasmic shuttling. A marked increase in nuclear PKD2 upon treatment with LMB also could be observed in other cell lines such as Panc-1 human pancreatic

cancer cells, COS-7, HEK293, and HeLa cells (Figure 5D; our unpublished data).

Role of the Regulatory Domains for Nucleocytoplasmic Shuttling of PKD2

The NES is a short leucine-rich sequence motif that binds Crm-1 and is necessary to mediate active nuclear export (Gorlich and Mattaj, 1996). NES transport motifs regulate the intracellular localization of a variety of proteins (Xu and Massague, 2004). It has been demonstrated that the PH domain in PKD1 contains a nuclear export signal (Rey *et al.*, 2001). The PKD2 mutant lacking the entire PH domain, EGFP-PKD2- ΔPH , was predominantly localized in the cytoplasm and around the nucleus of unstimulated, living AGS-B cells, but it did not accumulate in the nucleus (Figure 6A). Only upon incubation of AGS-B cells with gastrin and in the presence of LMB EGFP-PKD2- ΔPH was clearly detectable in the nucleus, resulting in a 10- and 21-fold increase in the nucleo/cytoplasmic fluorescence ratio, respectively. Thus, the subcellular localization of PKD2- ΔPH in unstimulated, living cells resembles closely that of wild-type PKD2 (Figure 6A), demonstrating that the PH domain in PKD2 does not contain a functional NES.

PKD2- $\Delta\text{C1a/1b}$ was found predominantly in the cytoplasm of unstimulated, living AGS-B cells. On gastrin treatment, EGFP-PKD2- $\Delta\text{C1a/1b}$ increased in the perinuclear area and to a lesser degree also in the nucleus. There was only a 2.7-fold increase in the nucleo/cytoplasmic fluorescence ratio of PKD2- $\Delta\text{C1a/1b}$ in response to gastrin in living cells. Even in the presence of LMB the nucleo/cytoplasmic fluorescence ratio of PKD2- $\Delta\text{C1a/1b}$ increased only by 4.6-fold. These data demonstrate that nuclear accumulation of PKD2 is, at least in part, prevented when the C1a/C1b domain is missing even under conditions that inhibit Crm-1-dependent nuclear export (Figure 6B). Therefore, this domain of PKD2 is likely to play a role in the regulation of nuclear entry.

The C1a/C1b Domain of PKD2 Contains a Functional Nuclear Localization Sequence

Importin- α is the nuclear import receptor that recognizes cargo proteins carrying conventional basic monopartite and bipartite NLSs (Chook and Blobel, 2001). First, we determined whether PKD2 can interact with importin- α . As shown in Figure 6C, importin- α coimmunoprecipitates with PKD2, suggesting that the nuclear uptake of PKD2 is mediated by importin- α . The binding of importin- α to PKD2 was constitutive (our unpublished data). However, the amount of importin- α that coprecipitated with PKD2 was markedly reduced when the C1a/C1b domain of the kinase was missing in accordance with our finding that nuclear accumulation of PKD2- $\Delta\text{C1a/C1b}$ is reduced compared with wild-type PKD2 (Figure 6C). Next, we examined whether the PKD2 sequence contains a potential NLS. NLSs are classified into various categories. The classical NLS exhibits a 4 residue pattern composed of four basic amino acids (K or R; pat4 NLS) (Hicks and Raikhel, 1995). A bipartite NLS is characterized by two basic residues, a 10-residue spacer, and another basic region consisting of at least three basic residues out of five residues (Robbins *et al.*, 1991). The zinc finger region of PKD1 contains a putative bipartite nuclear entry signal (Rey *et al.*, 2001). A similar signal sequence can be found in PKD2 at the C terminus of the C1a domain and in the linker region between C1a and C1b. The second part of this bipartite NLS in the linker region between C1a and C1b of PKD2 (¹⁹²RKRR¹⁹⁵) is itself a monopartite pat4 NLS (Figure 7). To determine the functional relevance of this

Figure 6. Role of the regulatory domain in nucleocytoplasmic shuttling of PKD2. AGS-B cells were plated in MatTek glass-bottomed dishes and subsequently transfected with vectors encoding EGFP-PKD2 (A, top) or EGFP-PKD2-ΔPH (A, bottom), EGFP-PKD2-ΔC1a/C1b (B), or EGFP-PKD2-K¹⁹³A/R¹⁹⁴G (C, bottom). Forty-eight hours after transfection cells were incubated with 10 ng/ml LMB or 100 nM gastrin (G) for 1 h. Controls received an equivalent amount of solvent (-). Representative images were captured in vivo and the relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*. C, top, HEK293 cells were transfected with FLAG-PKD2 (+) or Flag-PKD2-ΔC1a/C1b (+) as indicated. Forty-eight hours after transfection cells were lysed and subjected to immunoprecipitations using an anti-FLAG antibody (IP: Flag) followed by Western blotting with the anti-importin-α and the anti-FLAG antibody as indicated (WB). The positions of importin-α, Flag-PKD2, and Flag-PKD2-ΔC1a/C1b are indicated by arrowheads (▶).

sequence, the lysine residue 193 and the arginine residue 194 were changed to alanine and glycine, respectively. This mutant, EGFP-PKD2-K¹⁹³A/R¹⁹⁴G, was largely localized in the cytoplasm of unstimulated, living AGS-B cells. Interestingly, there was no detectable nuclear accumulation of EGFP-PKD2-K¹⁹³A/R¹⁹⁴G upon treatment of cells with gastrin and even in the presence of LMB despite the fact that PKD2-K¹⁹³A/R¹⁹⁴G exhibited a similar increase in catalytic activity as wild-type PKD2 in response to gastrin treatment of cells (our unpublished data). Consequently, there was no change in the nucleo/cytoplasmic fluorescence ratio of EGFP-PKD2-K¹⁹³A/R¹⁹⁴G in response to gastrin or LMB. Thus, the monopartite pat4 NLS in the linker region between the C1a

Figure 7. Potential nuclear localization sequence (NLS) within the C1a/C1b domain of PKD2. The C terminus of the C1a domain of PKD2 contains the first part of a putative bipartite NLS (yellow box). The second part of this bipartite NLS in the linker region between C1a and C1b (192RKRR195) can also be regarded as a pat4 NLS (blue). This sequence is highly conserved between PKD1, -2, and -3. For functional analysis of the pat4 NLS, a K193A/R194G mutant was generated (red).

and the C1b domain of PKD2 is indeed functional (Figure 6C).

The C1a Domain Is Required for Nuclear Export of PKD2 in Living Cells

The C1a domain of PKD2 contains the first 11 AA of the putative bipartite NLS at its C terminus (Figure 7). To analyze whether this part of the bipartite NLS also was relevant for nuclear import of PKD2, we examined the subcellular localization of the PKD2-ΔC1a mutant. Surprisingly, PKD2-ΔC1a was largely detectable in the nucleus already in unstimulated, living AGS-B cells. The nucleo/cytoplasmic fluorescence ratio of PKD2-ΔC1a in unstimulated AGS-B cells was well comparable to that of wild-type PKD2 in gastrin-treated cells. Consequently, incubation of cells with gastrin did not further increase nuclear accumulation of PKD2-ΔC1a (Figure 8A). Quantification of the nucleo/cytoplasmic fluorescence ratio of PKD2-ΔC1a revealed that only the complete block of Crm-1-dependent nuclear export by LMB was able to induce a slight, 1.5-fold increase in this ratio. Accordingly, binding of PKD2 to Crm-1 was markedly reduced when the C1a domain was missing (Figure 8A). Thus, the C1a domain contains a functional nuclear export signal rather than a nuclear localization sequence.

Determination of PKD2 and PKD2-ΔC1a Trafficking by FLIP

FLIP is a powerful approach to assess whether distinct cellular compartments are in equilibrium (White and Stelzer, 1999; Lippincott-Schwartz *et al.*, 2001). To compare the nuclear exchange of PKD2-ΔC1a and wild-type PKD2 with the cytoplasm, AGS-B cells were transfected with EGFP-PKD2-ΔC1a or EGFP-PKD2, respectively, and incubated with gastrin for 20 min. Subsequently, the cytoplasm was repetitively and selectively bleached, and we asked whether EGFP-PKD2-ΔC1a and EGFP-PKD2 fluorescence decreased in the nucleus as a consequence of nucleocytoplasmic shuttling. As shown in Figure 8B, the FLIP experiments revealed that there was a substantial loss of nuclear fluorescence of EGFP-PKD2 in living cells after bleaching of the cytoplasm. In contrast, there was very little loss of nuclear fluorescence of EGFP-PKD2-ΔC1a. Quantification of the data confirmed that EGFP-PKD2 does considerably move between the nucleus and the cytoplasm of AGS-B cells in response to gastrin. Half of the nuclear fluorescence of EGFP-PKD2 was lost 220 s after bleaching of the cytoplasm, and an apparent plateau of 10% of the initial fluorescence was reached after 700 s (Figure 8B, bottom). This kinetics is comparable to the loss of nuclear fluorescence of nucleoporin 98, which shows an

Figure 8. The C1a domain is required for nuclear export of PKD2. **A**, left, AGS-B cells were transfected with EGFP-PKD2-ΔC1a and plated in MatTek glass-bottomed dishes. Forty-eight hours after transfection, cells were incubated with 100 nM gastrin (G), 10 ng/ml LMB, or solvent (–) for 1 h. Representative images were captured in vivo, and the relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*. **A**, right, HEK293 cells were transfected with EGFP-PKD2 (EGFP-PKD2 +) or EGFP-PKD2-ΔC1a (EGFP-PKD2-ΔC1a +), lysed, and immunoprecipitated using an anti-GFP antibody followed by anti-Crm-1 or anti-GFP Western blotting as indicated. The right arrows indicate the position of EGFP-PKD2 and EGFP-PKD2-ΔC1a, respectively; the left arrow indicates the position of Crm-1. **B**, top, AGS-B cells were transfected with EGFP-PKD2 (left) or EGFP-PKD2-ΔC1a (right) and plated in MatTek glass-bottomed dishes. Forty-eight hours after transfection, cells were incubated with 100 nM gastrin (top) or 10 ng/ml LMB (bottom; our unpublished data) to induce nuclear translocation of PKD2. After 20 min of incubation at 37°C photobleaching and FLIP measurements were performed as described in *Materials and Methods*. An area of the cytoplasm (dotted line) was repeatedly bleached, and the loss of fluorescence in the nuclear region was determined by confocal microscopy. Bars, 5 μm . Bottom, graphical analysis of the FLIP data. There was an only 8% decrease of nuclear EGFP-PKD2 fluorescence intensity in the presence of LMB (gray curve). At the same time, nuclear EGFP-PKD2 fluorescence intensity decreased by 90% in the absence of LMB (red curve). In contrast, nuclear EGFP-PKD2-ΔC1a fluorescence intensity decreased only by ~20% (blue curve) compared with wild-type EGFP-PKD2 in the presence of LMB (gray curve).

NF1/2 of ~3 min and reaches an apparent final plateau of 8% of the initial fluorescence after ~20 min (Griffis *et al.*, 2002). When nuclear export was blocked by LMB, PKD2 did

Figure 9. A nuclear export signal (NES) in the C1a domain of PKD2. The C1a domain contains a potential NES. This sequence is highly conserved between PKD1, -2, and -3. For functional analysis of this sequence, we mutated Leu and Val to Ala at the positions 159 and 163, resulting in the NES-mutant PKD2-L¹⁵⁹/V¹⁶³A.

not shuttle at an appreciable rate (Figure 8B, bottom). Strikingly, nuclear fluorescence of EGFP-PKD2-ΔC1a decreased only by ~20% of the initial fluorescence compared with PKD2 in the presence of LMB. Thus, movement of PKD2 from the nucleus to the cytoplasm is substantially decreased when the C1a domain is missing. This further demonstrates that the C1a domain contains a functional NES.

NESs are characterized by a series of leucine or valine residues. According to these criteria, we identified a potential NES in the C1a domain of PKD2 between AA 159 and 167 (Figure 9). An EGFP-PKD2 mutant containing a dual amino acid exchange in this putative NES (EGFP-PKD2-L¹⁵⁹/V¹⁶³A) markedly accumulated in the nucleus of living AGS-B cells. There was a 3.3-fold increase in the nucleo/cytoplasmic fluorescence ratio of PKD2-L¹⁵⁹/V¹⁶³A compared with wild-type PKD2 (Figure 10A). Nuclear localization of PKD2-L¹⁵⁹/V¹⁶³A was confirmed by coimmunostaining using an anti-RNA polymerase II antibody (Figure 10A, bottom). PKD2-L¹⁵⁹/V¹⁶³A nuclear fluorescence intensity could not be enhanced by gastrin treatment of cells. However, the increase in catalytic activity of PKD2-L¹⁵⁹/V¹⁶³A upon incubation of cells with gastrin was similar to that of wild-type PKD2 (our unpublished data). The nucleo/cytoplasmic fluorescence ratio of this mutant was only further enhanced in the presence of leptomycin B (Figure 10A). Thus, L¹⁵⁹/V¹⁶³ in PKD2 is relevant for nucleocytoplasmic shuttling of the kinase.

Next, we examined whether PKD2-ΔC1b also could play a role in the regulation of nucleocytoplasmic shuttling of PKD2. As shown in Figure 10B, PKD2-ΔC1b was largely detectable in the cytoplasm of unstimulated, living AGS-B cells. This is in marked contrast to PKD2-ΔC1a. Furthermore, upon treatment of cells with gastrin, there was only a twofold increase in nuclear fluorescence intensity. This could suggest that this sequence contains a functional NLS. However, by blocking the Crm-1-dependent nuclear export with LMB we observed a marked, 14-fold increase in the nucleo/cytoplasmic fluorescence ratio of PKD2-ΔC1b, which was comparable to that of wild-type PKD2 (Figure 10B). Thus, nuclear entry is apparently not blocked upon deletion of the C1b domain of PKD2 and this mutant is still able to shuttle between the cytoplasm and the nucleus. Because PKD2-ΔC1b is not activated by gastrin treatment of cells (Figure 3A), the lack of catalytic activity could explain why gastrin treatment of AGS-B cells does not lead to an increase in nuclear fluorescence intensity of this mutant.

DISCUSSION

PKD2 is a novel member of the PKD family of serine/threonine kinases that is activated by pharmacological

Figure 10. Identification of a NES in the C1a domain of PKD2. A, top, AGS-B cells were transfected with EGFP-PKD2 (top) or EGFP-PKD2-L¹⁵⁹/V¹⁶³A (bottom) and plated in MatTek glass-bottomed dishes. Forty-eight hours after transfection cells were incubated with 100 nM gastrin (G), 10 ng/ml LMB, or solvent (–) for 1 h. Representative images were captured *in vivo* and the relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*. Bottom, a parallel culture of AGS-B cells transfected with EGFP-PKD2-L¹⁵⁹/V¹⁶³A was fixed and subsequently incubated with an anti-Pol-II antibody (red staining, Pol-II) followed by indirect immunofluorescence. Representative images were captured. The overlay of EGFP-PKD2-L¹⁵⁹/V¹⁶³A (green) and Pol-II immunostaining (red) is indicated by yellow. (B) AGS-B cells were transfected with EGFP PKD2-ΔC1b and incubated with gastrin or LMB as described above. Representative images were captured *in vivo* and the relative nuclear fluorescence intensity (F_{nuclear}) was calculated as described in *Materials and Methods*.

agents such as PDBu and by GPCRs by a PKC-dependent mechanism (Sturany *et al.*, 2001, 2002). Like the other PKD family members, the kinase contains a regulatory domain at the amino terminus comprising a duplex cysteine-rich domain, C1a/C1b, and a PH domain. Regulatory domains determine the basal activity of protein kinases and contribute to the regulation of their subcellular localization (Oancea *et al.*, 2003; Rykx *et al.*, 2003; Wang *et al.*, 2003). The aim of our study was to determine whether the regulatory domain in PKD2 has similar or distinct effects on the regulation of PDBu binding, catalytic activity and subcellular localization of the kinase. Our data show that there are certain similarities but also striking differences between PKD2 and the other members of the PKD family.

Role of the Regulatory Domain in Phorbol Ester Binding and Catalytic Activity of PKD2

We found that the PH domain of PKD2 does not bind PDBu and is a negative regulator of PKD2 kinase activity. Thus, the PH domain in PKD2 plays a similar role in the regulation of catalytic activity as the PH domain in PKD1 (Iglesias and Rozengurt, 1998). The C1a and the C1b domain of PKD2 differ substantially in their ability to mediate specific phorbol ester binding to the kinase. The C1b domain was found to be responsible for the majority of PDBu binding. Again, these data are in good agreement with the findings in PKD1 (Iglesias *et al.*, 1998). However, deletion of the entire cysteine-rich domain of PKD2 led only to a minor increase in the basal catalytic activity of PKD2 that could be further enhanced by treatment of cells with gastrin. In PKD1, deletion of the entire C1a/C1b domain, the C1a or the C1b motif, respectively, leads to maximum activation of the enzyme. Consequently, this domain also has been termed a negative regulator of PKD1 activity (Iglesias and Rozengurt, 1999). In PKD2, selective deletion of the PKD2-C1a domain did not increase basal catalytic activity and even more striking, deletion of the PKD2-C1b domain resulted in a complete loss of catalytic activity even upon incubation of cells with gastrin and despite phosphorylation of the mutant at Ser⁷⁰⁶/Ser⁷¹⁰ in the activation loop. Thus, despite a sequence homology between the C1a/C1b subdomains of PKD1 and 2, the C1b domain in PKD2 is a positive regulator of catalytic activity because lipid binding to this domain is required for its activation even if PKCs phosphorylate the critical serine residues in the activation loop.

PKD2 Shuttles between the Cytoplasm and the Nucleus

Regulatory domains also can modulate the subcellular localization of a protein. Our data show that PKD2 localizes to similar compartments as PKD1 and PKD3, the plasma membrane and the Golgi (Maeda *et al.*, 2001; Hausser *et al.*, 2002) but also the nucleus (Rey *et al.*, 2001, 2003b). It has been reported that activation of PKD2 does not induce its redistribution from the cytoplasm to the nucleus (Rey *et al.*, 2003a). Our data in living AGS-B cells but also in other cell lines indicate that PKD2 shuttles continuously between the cytoplasm and the nucleus by a Crm-1-dependent nuclear export mechanism. Furthermore, activation of GPCRs leads to a marked nuclear accumulation of PKD2. This finding does not exclude the fact that the precise localization of members of the PKD family may at least in part be cell type specific.

We did not detect a marked difference in the subcellular distribution of wild-type PKD2 and PKD2-ΔPH in AGS-B cells, suggesting that catalytic activity alone is not sufficient to trigger translocation of PKD2 to distinct subcellular compartments. Interestingly, there was no nuclear accumulation of PKD2-ΔPH, demonstrating that the PH domain of PKD2 has no obvious effect on nucleocytoplasmic shuttling. Thus, in marked contrast to PKD1 (Rey *et al.*, 2001), this domain does not mediate nuclear export of PKD2.

Functional Domains Involved in Nucleocytoplasmic Distribution of PKD2

The subcellular distribution of a PKD2-ΔC1a/C1b mutant was mainly cytoplasmic and hence similar to that of wild-type PKD2 in unstimulated AGS-B cells. However, the C1a/C1b domain contains a potential bipartite NLS at the C terminus of the C1a domain and a pat4 NLS in the linker region between the C1a and C1b domain. Mutation of the pat4 NLS (K¹⁹³A/R¹⁹⁴G) revealed that the lysine residue 193

and the arginine residue 194 are instrumental for efficient nuclear targeting of PKD2. Surprisingly, live cell imaging and our FLIP experiments revealed that C1a domain contains a functional NES. Here, the leucine residue 159 and the valine residue 163 were found to be crucial for nuclear export of PKD2. Thus, the potential NLS at the C terminus of the C1a subdomain does not seem to be functionally relevant and the NES is the predominant functional localization signal in this sequence. The NES motif as well as the bipartite NLS motif is highly conserved between PKD1, 2, and 3, suggesting that these domains could play similar roles in all members of the PKD family. It has been reported that the C1a domain of PKD1 does not interfere with nuclear import but slightly accumulated in the nucleus (Rey *et al.*, 2001). However, the precise role of the PKD1-C1a domain for nucleocytoplasmic shuttling has not been elucidated, and the sequence deleted to generate this mutant comprised the C1a domain including the linker region with the pat4 NLS (Rey *et al.*, 2001). We found no increase in nuclear PKD2- Δ C1b even in the presence of gastrin. However, in contrast to PKD1 (Rey *et al.*, 2001), there was a marked increase in nuclear PKD2- Δ C1b upon treatment of cells with leptomycin B. This suggests that PKD2- Δ C1b is still able to shuttle between the cytoplasm and the nucleus. Similar findings were obtained using a catalytically inactive PKD2-D⁶⁹⁵A mutant (our unpublished data). Therefore, the lack of nuclear accumulation of PKD2- Δ C1b is most likely due to the fact that this mutant cannot be activated by treatment of cells with gastrin.

In conclusion, examination of the regulatory domain of PKD2 revealed similarities, but also striking differences to the published data on PKD1 despite the high sequence homology. Our data demonstrate that the C1a/C1b domain in PKD2 has complex regulatory properties: The C1b domain is the major receptor for phorbol esters and plays a positive role in the regulation of catalytic activity, whereas the C1a domain and the linker region between C1a and C1b tightly regulate nucleocytoplasmic shuttling of the enzyme. It is suspected that in vivo, the substrate specificity of a kinase is likely to be determined both by affinity for its regulatory subunit that brings the kinase in proximity to the substrate and by subcellular localization. The ability to define the specific subcellular localization of PKD2 might enable us to gain further insight into compartment-specific signaling properties of this enzyme.

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