Protein kinase D1 mediates stimulation of DNA synthesis and proliferation in intestinal epithelial IEC-18 cells and in mouse intestinal crypts

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RUNNING TITLE: PKD1 enhances intestinal cell proliferation

ABSTRACT

We examined whether protein kinase D1 (PKD1), the founding member of a new protein kinase family, plays a critical role in intestinal epithelial cell proliferation. Our results demonstrate that PKD1 activation is sustained whereas that of PKD2 is transient in intestinal epithelial IEC-18 stimulated with the Gq-coupled receptor agonists angiotensin II or vasopressin. PKD1 gene silencing utilizing small interfering RNAs dramatically reduced DNA synthesis and cell proliferation in IEC-18 cells stimulated with Gq-coupled receptor agonists. To clarify the role of PKD1 in intestinal epithelial cell proliferation in vivo, we generated transgenic mice that express elevated PKD1 protein in the intestinal epithelium. Transgenic PKD1 exhibited constitutive catalytic activity and phosphorylation at the activation loop residues, Ser744 and Ser748 and on the autophosphorylation site, Ser916. In order to examine whether PKD1 expression stimulates intestinal cell proliferation, we determined the rate of crypt cell DNA synthesis by detection of 5-bromo-2-deoxyuridine (BrdU) incorporated into the nuclei of crypt cells of the ileum. Our results demonstrate a significant increase (p<0.005) in DNA synthesizing cells in the crypts of two independent lines of PKD1 transgenic mice as compared with non-transgenic littermates. Morphometric analysis showed a significant increase in the length and in the total number of cells per crypt in the transgenic PKD1 mice as compared with the non-transgenic littermates (p<0.01). Thus, transgenic PKD1 signaling increases the number of cells per crypt by stimulating the rate of crypt cell proliferation. Collectively, our results indicate that PKD1 plays a role in promoting cell proliferation in intestinal epithelial cells, both in vitro and in vivo.

INTRODUCTION

The mammalian intestine is covered by a single layer of epithelial cells that is renewed every 4-5 days along the crypt-villus axis (1). The high rate of cell turnover, driven by crypt cell proliferation, plays a fundamental role in the organization, maintenance and restoration of tissue integrity. It is recognized that the
sequential proliferation, lineage-specific differentiation, crypt-villus migration, and cell death of the epithelial cells of the intestinal mucosa is a tightly regulated process modulated by a broad range of regulatory peptides, differentiation signals, and luminal stimuli, including nutrients and pathogenic/commensal organisms (1-3). Despite its importance for understanding normal homeostasis, wound healing and the pathogenesis of human disease states, including inflammatory bowel diseases and colon cancer, the intracellular signal transduction mechanisms involved remain incompletely understood.

Protein kinase D1 (PKD1)1, the founding member of a new protein kinase family within the CAMK group and separate from the previously identified PKCs [reviewed in (4)], is attracting intense attention. PKD1 has been extensively studied in vitro, with regards to identifying the functions of its domains and the effect of cell signaling on its activity and sub-cellular localization (4). In unstimulated cells, PKD1 is in a state of low catalytic (kinase) activity maintained by autoinhibition mediated by the N-terminal domain, a region containing a repeat of cysteine-rich zinc finger-like motifs and a pleckstrin homology domain (4-7). PKD1 can be activated within intact cells by multiple stimuli acting through receptor-mediated pathways [reviewed in (4)]. Our own studies demonstrated rapid, PKC-dependent, PKD1 activation in response to G protein-coupled receptor (GPCR) agonists, including regulatory peptides (8-17) and bioactive lipids (12,18-20) that act through Gq, G12, Gi, and Rho (12,17-19,21,22), growth factors that act through tyrosine kinase receptors (8,23), cross-linking of B-cell receptor and T-cell receptor in B and T lymphocytes, respectively (24-26) and oxidative stress (27,28). The phosphorylation of Ser744 and Ser748 in the PKD1 activation loop (also referred as activation segment or T-loop) is critical for PKD1 activation (4,7,16,21,29). More recently, we showed that the rapid PKC-dependent PKD1 activation is followed by a sustained, PKC-independent, phase of catalytic activation and phosphorylation induced by stimulation of Gq-coupled receptor in COS-7 cells (30) and in 3T3 fibroblasts (31). Accumulating evidence implicates PKD1 in the regulation of multiple biological responses, including signal transduction (15,32-34), chromatin organization (35), gene expression (20,36,37), immune regulation (35) and cell survival, adhesion, motility, differentiation, DNA synthesis and proliferation [reviewed in Ref. (4)]. In fibroblasts, PKD1 overexpression potently enhanced long-term biological responses, including DNA synthesis and cell proliferation, induced by Gq-coupled receptor agonists (9,15,31). In contrast, neither the regulation nor the function of PKD1 in mediating proliferative responses in normal intestinal epithelial cells has been examined. Moreover, the role of PKD1 signaling in the replication of crypt intestinal epithelial cells in vivo has not been addressed. Indeed, very little is known about the biological role of PKD1 in normal epithelial cells of intact animals.

The experiments presented here were designed to define the regulation and function of PKD1 in intestinal epithelial cell proliferation, using IEC-18 and IEC-6 cells in culture (38,39). These cells, derived from cryptal cells of the small intestine, were used as model systems to examine the regulation of PKD1 activity and its role in DNA synthesis and proliferation of these intestinal epithelial cells (13,40,41). In order to evaluate the role of PKD1 in intact animals, we used transgenic expression of PKD1 in the mouse intestinal epithelium to determine the effect of its overexpression on cell proliferation and crypt architecture. Collectively, our results demonstrate that PKD1 promotes DNA synthesis and proliferation in intestinal epithelial cells both, in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

Stock cultures of IEC-18 and IEC-6 cells (38,39) were maintained at 37°C in
DMEM, supplemented with 5% fetal bovine serum in a humidified atmosphere containing 10% CO₂ and 90% air. For experimental purpose, IEC-18 or IEC-6 cells were seeded in 35 mm dishes at a density of 2 × 10⁵ cells/dish the day before transfection. Our previous studies established that these cells express Gq-coupled receptors for ANG II and vasopressin (13,40-44).

**Immunoblotting and detection of PKD and MARCKS**

Confluent IEC-18 cells were lysed in 2x SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (20 mM Tris/HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol) and boiled for 10 min. After SDS-PAGE, proteins were transferred to Immobilon-P membranes. The transfer was carried out at 100 V, 0.4 A at 4°C for 4 h using a Bio-Rad transfer apparatus. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, 0.01% SDS, and 20% CH₃OH. For detection of proteins, membranes were blocked using 5% nonfat dried milk in PBS (pH 7.2) and then incubated for at least 2 h with the desired antibodies diluted in PBS containing 3% nonfat dried milk. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat antibodies. The phosphospecific antibodies used were as follows: the phospho PKD polyclonal antibodies pS⁹¹⁶, pS⁷⁴⁴ and pS⁷⁴⁸ detect PKD only when it is phosphorylated on Ser⁹¹⁶, Ser⁷⁴⁴ or Ser⁷⁴⁸; the phospho MARCKS polyclonal antibody is specific to MARCKS only when it is phosphorylated on Ser¹⁵² and Ser¹⁵⁶ (pMARCKS). Autoluminograms were scanned using a GS-710 scanner (Bio-Rad), and the labeled bands were quantified using the Quantity One software program (Bio-Rad).

**Immunoprecipitation and kinase assay of PKD**

**Immunoprecipitations.** Confluent IEC-18 cells were washed twice with DMEM and equilibrated in 5 ml of the same medium at 37 °C for 1-2 h. Some dishes were treated with various pharmacological agents during this equilibration period or with agonists for different times at the end of this period, as indicated in the corresponding figure legends. Cells were lysed in buffer A containing 50 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 100 µg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (Pefabloc) and 1% Triton X-100. PKD was immunoprecipitated with the PKD (PKD C-20) antiserum (1 µg/ml) raised against the C-terminal region of PKD1 (Santa Cruz). The immune complexes were recovered using protein-A coupled to agarose.

**Syntide-2 in vitro kinase assays.** Immune complexes 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. Reactions were initiated by combining 20 μl of immune complexes with 10 μl of a phosphorylation mixture containing 100 μM [γ-³²P]-ATP and syntide-2 at a final concentration of 2.5 mg/ml in kinase buffer. (final reaction volume, 30 μl), and transferred to a water bath at 30 °C for 10 min. Reactions were terminated by adding 100 μl of 75 mM H₃PO₄, and 75 μl of the mixed supernatant was spotted to Whatman P-81 phosphocellulose paper. Papers were washed thoroughly in 75 μM H₃PO₄, dried, and radioactivity incorporated into peptides was determined by detection of Cerenkov radiation in a scintillation counter.

**Assay of DNA Synthesis**

Confluent cultures of IEC-18 cells were washed twice with DMEM and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing various agonists as described in the figure legends. After 18 h of incubation at 37 °C, [³H]-thymidine (0.2 μCi/ml, 1 μM) was added to the cultures for 6 h, the cultures were then washed twice with PBS and incubated in 5% trichloroacetic acid at 4 °C for 20 min to remove acid-soluble radioactivity, washed.
with ethanol, and solubilized in 1 ml of 2% Na$_2$CO$_3$, 0.1 M NaOH. The acid-insoluble radioactivity was determined by scintillation counting in 6 ml of Beckman Readysafe.

**Knockdown of PKD1 or PKD2 levels via siRNA transfection**

The pooled siRNA duplexes were purchased from either Dharmacon (Lafayette, CO) or Santa Cruz (Santa Cruz, CA). PKD1 siRNA pools were designed to target the mRNA of mouse PKD1 (GenBank accession number NM_008858) and consists of four different duplexes from (Dharmacon): oligo1, GAAGAGAUGUAGCUAUUAAUU; oligo2, GAAAGAGUGUUGUGUUAUU; oligo3, CAUAAGAGAUGUGCAUUUAUU; oligo4, CAGCGAAUGUAGUAGUAUUAUU or three different duplexes from (Santa Cruz) oligo1, CUCUCUUUCGUAUCAUAt; oligo2, GUGAGCAUUUCCGUUUCAAtt; oligo3, GAAGCAUGAUCUUAUCAtt. PKD2 siRNA consisted of a pool of three duplexes (Santa Cruz): oligo1, CCAUUGAGUUGGACAAGAAtt; oligo2, CACCUGCACCUCAGAACAtt; oligo3, CCAGAAAUGAACUCUUGUAtt. For siRNA transfection the reverse transfection method was used, the siRNA pool was mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and added to 35mm dishes. IEC-18 cells were then plated on top of the siRNA/Lipofectamine RNAiMAX complex at a density of 1 x 10$^5$ cells/35 mm dish. Control transfections were carried out with Dharmacon siCONTROL non-targeting siRNA four-oligo pool (Sc) (catalog number D-001206-13). Four days after transfection, cells were used for experiments and subsequent Western blot analysis.

**Generation of PKD1 transgenic mice**

In order to generate transgenic mice that express elevated PKD protein in the intestinal epithelium, we used the rat fatty acid-binding protein (FABP) promoter (−596 to +21; kindly provided by Dr. Jeffrey Gordon, Washington University, St. Louis, MO) which has been well characterized to target transgene expression to proliferating and non-proliferating epithelial cells of these regions of the intestine of FVB/N mice (45). The FABP promoter was fused to the cDNA for mouse PKD1 (+40 to +2910) and the bovine growth hormone polyadenylation signal sequence using conventional cloning methods. A schematic diagram of the transgene construct (confirmed by sequencing) is shown in Fig. 4, A. The transgene was excised by digestion with EcoRI and XhoI, gel purified and microinjected into FVB/N mouse oocytes. The integrated transgene was detected by PCR using genomic tail DNA and specific oligonucleotide primers F12 (sense, 5’-TATAACCGCTCTCTTGAC), corresponding to the PKD1 portion of the transgene, and BGHR (antisense, 5’-ACTCAGACAATGC), corresponding to the bovine growth hormone polyadenylation sequence. We identified transgenic founder mice by PCR using genomic tail DNA and specific primers to detect the integrated transgene sequence as a product of 600bp (Fig. 4, B). Founder mice were mated with wild type FVB/N mice and two PKD1 transgenic lines (409 and 417) were established, propagated and used in subsequent experiments. All mice were housed in specific pathogen-free barrier facilities, maintained on a 12-h light, 12-h dark cycle, and fed a standard autoclavable rodent diet.

**Determination of intestinal cell proliferation**

The number of proliferating cells was detected by immunoperoxidase staining for the thymidine analog 5-bromo-2’-deoxyuridine (BrdU). Gender and age-matched mice, (PKD1 transgenic and non-transgenic littermates), were injected intraperitoneally with BrdU (100µg/g body weight). After 3h, the mice were anesthetized with halothane and cardioperfused with PBS followed by 4% paraformaldehyde in PBS and the ileum was removed and processed as described above. Sections (4µm) of paraffin embedded ileal tissue were deparaffinized and stained for...
BrdU incorporation using BrdU staining kit (BrdU In-Situ Detection Kit II #551321, BD Pharmingen, San Jose, CA) according to the manufacturer’s instructions. The proportion of BrdU-positive cells was determined at high magnification under light microscopy. Crypt cell proliferation was expressed as the percentage of BrdU-labeled cells per 100 crypt cells and at least 20 full-length, well-oriented ileal crypts per mouse were counted.

**Intestinal morphometry**

Hematoxylin- and eosin-stained histological sections were analyzed to determine the effect of transgenic PKD1 expression on tissue architecture. Briefly, 20 full-length, longitudinally cut crypts from each animal were analyzed for height (µm) and number of cells per crypt height. Cross-section of crypts (20/mouse) were used to determine the average crypt diameter (µm) and circumference (in number of cells). These data were used to calculate cell size (crypt height in µm/crypt height in cell number) and estimate the total cells per crypt (mean cells per crypt column × mean crypt circumference).

**Materials**

[^32P]-ATP (specific activity, 4500 Ci/mmol) was obtained from Perkin Elmer Waltham, Massachusetts. Horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were from GE Healthcare. Protein A-agarose and Pefabloc were from Roche (US). Angiotensin II, vasopressin, EGF, and GFI were obtained from Sigma, St. Louis MO. G66983 was from Calbiochem, La Jolla, CA. We used two different antibodies to detect the phosphorylated state of Ser744 and Ser748 in the PKD activation loop. One antibody (anti-pS744/pS748), obtained from Cell Signaling Technology, Beverly, MA, was raised against a peptide phosphorylated on serines equivalent to Ser744 and Ser748 of PKD1 but predominantly detects the phosphorylated state of Ser744, as shown originally in our laboratory (16). A second antibody, obtained from Abcam (ab17945), detects the phosphorylated state of Ser748. The specificity of this antibody was confirmed in our recent study using PKD1 with Ser744 and Ser748 mutated to non-phosphorylatable alanines (30). The two different antibodies were used that recognize the autoprophosphorylation site Ser916 (PKD1) or Ser876 (PKD2), these were purchased from Millipore (04-787) and Abcam (ab59417). An antibody that detects the C-terminal region of PKD1 and PKD2 (C-20) was from Santa Cruz. All other reagents were from standard suppliers or as described in the text, and were of the highest grade commercially available.

**RESULTS and DISCUSSION**

**GPCR agonists induce PKD1 and PKD2 activation with different kinetics in IEC-18 cells.**

In order to determine the kinetics of PKD activation by agonists that stimulate endogenously expressed Gq-coupled receptors in intestinal epithelial IEC-18 cells, cultures of these cells were stimulated for various times (2.5-240 min) with either ANG II (Fig. 1A) or vasopressin (Fig.1B). Cell lysates were analyzed by SDS-PAGE followed by Western blotting using an antibody that detects the autophosphorylated state of PKD1 at Ser916 and PKD2 at Ser876. Prior to stimulation, PKD1 and PKD2 exhibit a very low level of autophosphorylation in IEC-18 cells (Fig. 1A, B). Stimulation of these cells for 2.5 min induced a striking increase in the phosphorylation of a doublet band corresponding to the apparent molecular mass of PKD1 autophosphorylated on Ser916 (upper band, 110 kDa) and PKD2 autophosphorylated on Ser876 (lower band, 105 kDa), respectively. Interestingly, the kinetics of autophosphorylation of PKD1 and PKD2 were strikingly different, regardless of the agonist used. The phosphorylation of PKD2 declined rapidly toward base-line levels whereas that of PKD1 remained elevated for up to 4 h (Fig. 1A and B). In other experiments, PKD1 phosphorylation on Ser916 was still increased
above baseline levels even after 24h of ANG II stimulation (results not shown).

In order to verify that the doublet band detected in the IEC-18 lysates (shown in Fig. 1) corresponds to PKD1 and PKD2, we knockdown the expression of each isoform using siRNAs that target specifically either PKD1 or PKD2. Then, the cells were stimulated for 5, 30 and 120 min with 50 nM ANG II and Western blot analysis of the lysates was performed using an antibody that detects the autophosphorylated state of PKD1 at Ser916 and PKD2 at Ser876. As shown in Fig. 1 C, transfection of siRNA targeting PKD1 produced striking knockdown of the upper band without altering the intensity or kinetics of the lower band, as compared with the non-targeting control. Reciprocally, transfection of siRNA targeting PKD2 knockdown the expression of the lower band without affecting the intensity or kinetics of the upper band. These results confirmed the identity of the doublet band detected in lysates of IEC-18 cells, as PKD1 (110 kDa band) and PKD2 (105 kDa band) and imply that PKD1 activation is sustained whereas that of PKD2 is transient.

The amino acid sequence corresponding to the activation loop of PKD1 and PKD2 is identical and consequently, an antibody directed against the phosphorylated state of the residues of the activation loop detects both PKD isoforms. In order to verify that the kinetics of activation of PKD1 differs from that of PKD2 in IEC-18 cells, we examined the phosphorylation of the activation loop of these isoforms using an antibody that specifically detects the phosphorylated state of Ser744 and Ser748 in PKD1 and Ser706 and Ser710 in PKD2 (7,16,26). We also monitored autophosphorylation of PKD1 on Ser916 and PKD2 on Ser876, as described above. In agreement with previous results, these antibodies detected a doublet corresponding to PKD1 and PKD2 in cells stimulated for 10 min. Treatment with GFI profoundly inhibited activation loop phosphorylation and autophosphorylation of both isoforms induced by stimulation with agonist for 10 min (Fig. 2 A).

A salient feature of the results presented in Fig. 2 A is that treatment with GFI did not prevent sustained PKD1 activation induced by vasopressin stimulation for 180 min, as shown by Ser916 autophosphorylation and by in vitro kinase assays (see below).
Furthermore, GFI did not prevent the phosphorylation of the PKD1 activation loop residue Ser\textsuperscript{748} but reduced the phosphorylation on Ser\textsuperscript{744}. As a control of the effectiveness of GF I at later times of incubation, we verified that this inhibitor completely blocked the phosphorylation of the prominent PKC substrate MARCKS (47-50) on Ser\textsuperscript{152/156}, either at early or late times of agonist stimulation. We also demonstrated that the preferential PKC inhibitor Go6983 (2.5 μM), which inhibits all isoforms of the PKC family but not PKD1 (51), also prevented early but not late PKD1 multi-site phosphorylation in response to ANG II in IEC-18 cells (Fig. 2 B). These experiments indicate that GPCR agonists stimulated late PKD1 activation in IEC-18 cells when PKCs in the same cells were inactive.

In order to corroborate biphasic PKD activation in intestinal epithelial cells, we also determined PKD catalytic activity in immunocomplexes by its ability to phosphorylate syntide-2, an exogenous substrate for PKDs (7,17,52,53). As shown in Fig. 2C, stimulation of IEC-18 cells with vasopressin caused a rapid and persistent increase in PKD1 catalytic activation. Treatment with GFI markedly inhibited vasopressin-induced PKD1 activation at the early time point (10 min) by ~70% but did not inhibit catalytic activation at the later time of incubation (180 min). These results support the notion that sustained PKD1 activation shifts from PKC-dependent to PKC-independent in GPCR-stimulated intestinal epithelial IEC-18 cells.

We also determined the localization of active PKD (i.e. PKD phosphorylated at Ser\textsuperscript{916}) after 3 or 120 min of ANGII or vasopressin stimulation, i.e. during the PKC-dependent and PKC-independent phases of PKD1 stimulation. As shown in Supplementary Fig. S3, active PKD was primarily detected at the cell membrane after 3 min of stimulation with the GPCR agonists. In contrast, most PKD1 phosphorylated at Ser\textsuperscript{916} was detected in the cytosol and nucleus rather than in the plasma membrane in IEC-18 cells stained after 120 min of GPCR stimulation.

In additional experiments, we also examined the role of PKC in PKD activation in cultures of intestinal epithelial IEC-6 cells. As with IEC-18 cells, PKD1 and PKD2 displayed different activation kinetics and treatment with GFI blocked autophosphorylation of PKD1 and PKD2 induced by ANG II stimulation for 10 min but did not prevent sustained PKD1 activation induced by stimulation with this agonist for 180 min (Fig. 2, D). Furthermore, GFI did not prevent PKD1 phosphorylation on Ser\textsuperscript{748} and reduced (but did not eliminate) the phosphorylation on Ser\textsuperscript{744}. These results indicate that GPCR agonists stimulate sustained PKD1 activation via a sequential mechanism consisting of an early PKC-dependent phase and a late PKC-independent phase in crypt-derived intestinal epithelial IEC-18 and IEC-6 cells. The sustained activation of PKD1 raised the possibility that this PKD isoform was preferentially associated to long-term cellular responses, including stimulation of DNA synthesis and cell proliferation in response to agonist stimulation.

**Knockdown of PKD1 selectively abrogates c-Fos accumulation, DNA synthesis and cell proliferation induced by GPCR agonists in IEC-18 cells.**

To determine the role of endogenous PKD1 in GPCR-induced mitogenesis in intestinal epithelial IEC-18 cells, we depleted its expression using siRNAs that target specifically PKD1. In agreement with the results in Fig. 1C, transfected siRNAs targeting PKD1 produced striking knockdown of PKD1, as revealed in Fig. 3 A by Western blot analysis of cell lysates with an antibody that detects the C-terminal region of PKD1 and PKD2 (PKD C-20). Densitometric scanning of the doublet of immunoreactive bands obtained in 6 independent experiments revealed that siRNA targeting PKD1 reduced its protein expression by ~90% (bars in Fig. 3,
A). In contrast, the intensity of the PKD2 band was not changed.

Exploiting the efficiency of siRNA-mediated PKD1 depletion, we determined the role of PKD1 in the stimulation of DNA synthesis and cell proliferation by the GPCR agonists vasopressin and ANG II. As shown in Fig. 3 B, stimulation of IEC-18 cells with increasing concentrations of these agonists induced [3H] thymidine incorporation into DNA in a concentration-dependent manner. The maximal DNA synthesis induced by these agonists was comparable to that elicited by 50 ng/ml EGF (shown for comparison). The salient feature of the results is that PKD1 knockdown prevented the increase in DNA synthesis and cell number induced by each of these GPCR agonists in IEC-18 cells (Fig. 3 C). In addition, PKD1 knockdown abrogated c-Fos accumulation induced by vasopressin in IEC-18 cells (Fig. 3 C, inset). Similarly, knockdown of PKD1 protein via a siRNA targeting a different sequence of the PKD1 gene, markedly attenuated stimulation of DNA synthesis induced by either ANG II or vasopressin in IEC-18 cells (Supplementary Fig. S4).

Collectively, these results demonstrate that persistent PKD1 activation plays a key role in mediating GPCR-induced cell proliferation in cultured intestinal epithelial cells.

**Catalytic activity and phosphorylation of PKD1 extracted from intestinal mucosa from control and PKD1 transgenic mice.**

In order to examine the effect of PKD1 on intestinal epithelial cell proliferation in vivo, we generated transgenic mice that express elevated PKD1 protein in the intestinal epithelium. For this purpose, we used a rat fatty acid-binding protein (FABP) promoter, which has been well characterized to target transgene expression to epithelial cells of the intestine of FVB/N mice (45). Two independent PKD1 transgenic mouse lines, 409 and 417 were used in subsequent experiments. As shown in Fig. 4 A, the level of PKD1 protein was markedly increased in epithelial cell lysates of the distal small intestine (ileum) of PKD1 transgenic mice (lines 409 and 417), as compared with non-transgenic littermate mice (NTg). Immunoreactive bands corresponding to PKD1 and PKD2 can be seen in extracts of NTg mice after longer exposures (results not shown). In other experiments, striking PKD1 overexpression was also verified after immunoprecipitation of intestinal lysates with PKD C-20. The detection of the immunoreactive transgenic PKD1 band was extinguished by inclusion of the immunizing peptide during the immunoprecipitation (Supplementary Fig. S5).

In agreement with the Western blot analysis shown in Fig. 4 B and Fig. S5, histological analysis of ileum of transgenic and non-transgenic littermates showed increased immunostaining for PKD1 in the epithelial cells of the crypts and villus when compared with non transgenic controls (Fig. 4 C). Interestingly, many cells in the crypts of transgenic mice displayed nuclear PKD1 staining. The nuclear localization of PKD1 suggested that the enzyme is active in the epithelium, consistent with the nuclear localization of activated PKD1 in IEC-18 cells (Fig. S3) and other cells.

To determine whether transgenic PKD1 is functional, we determined the catalytic activity and phosphorylation of PKD1 immunoprecipitated from scraped intestinal extracts obtained from transgenic and nontransgenic mice. The catalytic activity of PKD1 eluted from immunocomplexes was measured by in vitro kinase assays, using syntide-2 as a substrate in the absence or in the presence of lipid activators phosphatidylserine (PS) and PDBu (52). As a positive control, we also examined catalytic activity of PKD1 from lung extracts, a tissue previously shown to contain a high level of PKD1 that was stimulated by lipid activators. Furthermore, we determined whether PKD1 extracted from the ileal epithelium of transgenic mice is phosphorylated at the activation loop residues (Ser744 and Ser748) and at the autophosphorylation site Ser916.

As shown in Fig. 4D, PKD1 catalytic
activity was strikingly higher in extracts from transgenic mice as compared with non-transgenic mice. Interestingly, these results also indicate that PKD1 was in an active state since its high basal activity was only slightly further enhanced by addition of PS and PDBu. In contrast, PKD1 isolated from the lungs of the same mice using an identical procedure of lysis, immunoprecipitation and elution was markedly stimulated by addition of the lipid activators (PS and PDBu) to the incubation mixture (insert, Fig. 4D). These results reinforce the conclusion that the high basal activity of PKD1 over-expressed in the intestinal epithelium is in an active state. In agreement with this conclusion, transgenic PKD1 from lines 409 and 417 exhibited phosphorylation at the activation loop residues, Ser\(^{744}\) and Ser\(^{748}\) and on the autophosphorylation site, Ser\(^{916}\) (Fig. 4E). Furthermore, PKD1 was detected in the nucleus of crypt epithelial cells, consistent with the nuclear localization of active PKD1. Collectively, these results indicate that transgenic PKD1 is over-expressed and active in the intestinal epithelium and phosphorylated on residues that reflect its activation state, as has been identified in cells in culture, including intestinal epithelial cells (in Figs. 1 and 2).

**Transgenic expression of PKD increases the rate of crypt cell proliferation**

In the lining of the mammalian small intestine, dividing cells are restricted to the crypts. The progeny of these dividing cells migrate bi-directionally and give rise to Paneth cells at the base of the crypts and to enterocytes, goblet cells, and enteroendocrine cells that reside on the villi where no further proliferation occurs and the cells are fully differentiated [reviewed in (54)]. In order to assess whether PKD1 expression stimulates intestinal cell proliferation, we determined the rate of crypt cell DNA synthesis by immunohistochemical detection of BrdU incorporated into the cell nuclei of cells of the ileum, the region of the intestine from where IEC-18 cells were originated. Representative histological sections showing BrdU incorporated into the cell nuclei of crypt cells of the ileum of PKD1 transgenic mice (line 409) and non-transgenic littermates are illustrated in Fig. 5, A. The percentage of BrdU-labeled crypt cells in the control (open bars) and in PKD1 transgenic mice (closed bars) was quantified (Fig. 5, B). The results show a highly statistically significant increase (p<0.005) in DNA synthesizing cells in the crypts of the PKD1 transgenic mice as compared with non-transgenic littermates. Similarly, BrdU immunostaining corroborated increased proliferative activity in the crypts of the ileum of the second line of PKD1 transgenic mice (line 417; Fig. 5, C). In crypts from non-transgenic mice, BrdU-positive cells were prominent in a proliferation zone containing the transit amplifying cells. The proliferating cells in the crypts of PKD1 transgenic mice were distributed more widely and also localized in the bottom of the crypt to positions consistent with those corresponding to columnar basal cells regarded as intestinal stem cells (Fig. 5, D). We found a statistically significant increase (p<0.01) in DNA synthesizing columnar basal cells in the crypts of the PKD1 transgenic mice as compared with non-transgenic littermates (Fig. 5, E). These results support the hypothesis that PKD1 stimulates mitogenic signaling in crypt intestinal epithelial cells.

**Morphometric analysis of intestinal crypts in PKD transgenic and non-transgenic mice**

In the intestine, normal cell numbers are maintained by balancing rates of cell proliferation, differentiation, migration and apoptosis. Since our results indicate that transgenic expression of PKD1 markedly increases the rate of crypt cell proliferation (Fig. 6), we determined whether transgenic PKD1 leads to a change in tissue architecture, manifested by an increase in the size and total number of epithelial cells in the crypts.

To examine this possibility we measured crypt height (in micrometer and cell number) and crypt circumference (in
micrometer and cell number) in histological sections of control and PKD1 transgenic mice. The data was used to calculate the size of individual cells and the total number of cells per crypt. Neither crypt circumference nor size of individual cells showed a significant change (Fig. 6, upper panels). In contrast, our results demonstrated a significant increase (p<0.01) in the depth (measured either in μm or in number of cells) and in the total number of cells per crypt in the transgenic PKD1 mice as compared with the nontransgenic littermates (Fig. 6, lower panels). These results indicate that the expression of the PKD1 transgene led to a marked increase (44%) in the total number of intestinal epithelial cells per crypt.

CONCLUDING REMARKS

The sequential proliferation, lineage-specific differentiation, crypt-villus migration, and cell death of the epithelial cells of the intestinal mucosa is a highly regulated process involving a broad range of regulatory peptides, differentiation signals, and luminal stimuli. Indeed, the intestinal epithelium is an exquisite model to elucidate the role of signal transduction pathways during epithelial cell proliferation and differentiation (1). Furthermore, repeated damage and substantial injury of the intestinal surface, a key feature of inflammatory bowel diseases, require constant proliferative repair of the epithelium. Increased epithelial cellular proliferation is a significant risk factor for development of colon cancer (55). Despite its importance for understanding normal homeostasis, pathogenesis of disease states and identification of molecular targets for therapeutic intervention, the intracellular signal transduction mechanisms involved remain incompletely understood.

In the present study, we examined whether PKD1, the founding member of a new protein kinase family, plays a role in intestinal epithelial cell proliferation using both, epithelial cells in culture and a novel PKD1 transgenic mouse model. Our results demonstrate that stimulation of crypt-derived rat epithelial IEC-18 cells with the mitogenic Gq-coupled receptor agonists ANG II and vasopressin induced a rapid increase in PKD1 and PKD2 autophosphorylation. Interestingly, PKD2 autophosphorylation declined rapidly toward base-line levels while PKD1 activation remained elevated for many hours. These results indicated that agonist-induced PKD2 activation is transient whereas that of PKD1 is sustained within the same intracellular environment. Further studies demonstrated that the Gq-coupled receptor agonists induce PKD1 activation loop phosphorylation (e.g. Ser^748) via sequential PKC-dependent and PKC-independent phases. Thus, PKD1 can be activated persistently in intestinal epithelial IEC-18 cells through a PKC-independent pathway.

Given that GPCR agonists induce sustained PKD1 activation and act as potent mitogens for IEC-18 epithelial cells, we next examined the role of this isoform in mediating stimulation of DNA synthesis and proliferation of these cells. We found that selective siRNA-mediated knockdown of PKD1 dramatically reduced GPCR-induced DNA synthesis and cell proliferation in IEC-18 cells. Knockdown of PKD1 also prevented agonist-induced c-Fos accumulation in these cells. Collectively, these results identify a pathway by which agonists of endogenously expressed Gq-coupled receptors, such as ANG II and vasopressin (13,40-42), induce sustained PKD1 activation leading to c-Fos accumulation, DNA synthesis and cell division in intestinal epithelial IEC-18 cells. Given that these cells (38,39), derived from cryptal cells of the ileum, have been used extensively as a model system to examine migration, proliferation and differentiation (13,40-44), our results raised the attractive possibility that PKD1 signaling increases the rate of intestinal epithelial cell proliferation in vivo. However, nothing was known about the function of PKD1 signaling in the proliferation of intestinal epithelial crypt cells of intact animals.

As a first step to determine whether PKD1 signaling stimulates intestinal epithelial
cell proliferation in vivo, we generated transgenic mice that express elevated PKD1 protein in intestinal epithelial cells. Our results demonstrate a significant increase in the proportion of DNA synthesizing cells seen in the crypts of the PKD1 transgenic mice as compared with non-transgenic littermates. The proliferating cells in the crypt were localized to positions consistent with those corresponding to transit amplifying and columnar basal stem cells. Morphometric analysis showed a significant increase in the length and in the total number of cells per crypt in the transgenic PKD1 mice as compared with the non-transgenic littermates. The results indicate that transgenic PKD1 signaling increases the number of cells per crypt by stimulating the rate of intestinal crypt cell proliferation.

In conclusion, our results with crypt-derived rat epithelial IEC-18 cells indicate that PKD1 activation mediates GPCR-induced DNA synthesis and cell proliferation. Transgenic mice that express elevated PKD1 protein in intestinal cells, display a significant increase in DNA synthesizing cells in their intestinal crypts. Morphometric analysis demonstrated a significant increase in the length and in the total number of cells per crypt in the transgenic PKD1 mice, implying that PKD1 increases the number of cells per crypt by stimulating the rate of crypt cell proliferation. Collectively, our results support the notion that PKD1 signaling is a novel element in the pathways leading to proliferation of intestinal epithelial cells, both in vitro and in vivo.

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Footnotes:  
1. The abbreviations used are: PKC, protein kinase C; PKD1, protein kinase D1 isoform; PKD2, protein kinase D2 isoform; ANG II, angiotensin II; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BrdU, 5-bromo-2'-deoxyuridine; PAGE, polyacrylamide gel electrophoresis; CBC, columnar basal cells.

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**FIGURE LEGENDS**

**Figure 1. Time-course of induced PKD autophosphorylation induced by ANGII and vasopressin in intestinal epithelial cells.** IEC-18 cells were incubated in the presence 50 nM ANGII (Panel A) or vasopressin 50 nM (Panel B) for the indicated times. The cultures were then lysed with 2×SDS–PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with phospho PKD pSer\textsuperscript{916} polyclonal antibody. Equivalent loading of the gel was verified using Western blot analysis with an antibody directed against the C-terminal region of PKD (PKD-C20). Shown here are representative autoluminograms; similar results were obtained in four independent experiments. Similar results were obtained with a different antibody that detects the phosphorylated state of PKD1 and PKD2 at the C-terminal autophosphorylated site. **Panel C.** IEC-18 cells were transfected with either non-targeting negative control (N.Targ.) or 50 nM siRNA targeting PKD1 (PKD1 siRNA) or PKD2 (PKD2 siRNA). After 4 days when the cells were confluent and quiescent, the cultures were washed and incubated with 50 nM ANGII for the indicated times. The cultures were then lysed with 2×SDS–PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with phospho PKD pSer\textsuperscript{916} polyclonal antibody and PKD C20.

**Figure 2. PKC-dependent and PKC-independent PKD activation in intestinal epithelial cells stimulated with vasopressin and ANGII.** Panel A, IEC-18 cells were incubated in the presence (+) or in the absence (-) of 3.5 μM GF1 for 1 h prior to stimulation with 50 nM vasopressin for either 10 min or 180 min, as indicated. Panel B, IEC-18 cells were incubated in the presence (+) or in the absence (-) of 2.5 μM Go6983 for 1 h prior to stimulation with 50 nM ANGII for either 10 min or 180 min, as indicated. Panel C, PKD activity was measured by syntide-2 phosphorylation in immune complexes from lysates of cells that were incubated in the presence (filled bars) or in the absence (open bars) of 3.5 μM GF 109203X for 1 h prior stimulation with 50 nM vasopressin for the indicated times. The values shown are from at least two independent experiments. Panel D, IEC-6 cells were incubated in the presence (+) or in the absence (-) of 3.5 μM GF1 for 1 h prior to stimulation with 50 nM ANG II for either 10 min or 180 min, as indicated. In all cases, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with the following antibodies phospho PKD pSer\textsuperscript{916}, pSer\textsuperscript{744}, pSer\textsuperscript{748} and PKD-C20 to verify equal loading and phospho MARCKS in panel A. The results shown here are representative autoluminograms; similar results were obtained in at least three independent experiments.

**Figure 3. Panel A. Selective knockdown of endogenous PKD1 by siRNA targeting PKD1 in IEC-18 cells.** Cells were transfected with either non-targeting negative control (N.Targ.) or 75 nM PKD siRNA (PKD1), as indicated (Left). The cells were lysed and PKD protein expression was assessed by Western blotting using the anti-PKD C-20 antibody. Shown here is a representative autoluminogram; similar results were obtained in six independent experiments. Autoluminograms were quantified by densitometric scanning. The results shown in the bars (Right) are the mean ± S.E.M; n=6 and are expressed as percentage of the maximum level of PKD1 in non-targeting negative control cells (open bar). PKD1 expression was reduced by 88-92% (solid bar). **Panel B. ANGII and vasopressin stimulate DNA synthesis in IEC-18 cells in a concentration-dependent manner.** Confluent and quiescent cultures of IEC-18 were washed and incubated at 37°C in 2 ml of DMEM/Waymouth’s medium containing [\textsuperscript{3}H]-thymidine in the
presence of angiotensin II (ANGII) or vasopressin (VP), at the indicated concentrations. Parallel cultures received 50ng/ml EGF (solid bar) and it is shown for comparison. Results are expressed as a percentage mean ± S.E. (n=3) of the maximal stimulation obtained (38 x 10^{-3} cpm/culture).

**Panel C** Knockdown of endogenous PKD1 attenuates DNA synthesis and cell proliferation in response to vasopressin and ANGII (Lower left), IEC-18 cells were transfected with either non-targeting negative control (open bars) or 75 nM siRNA targeting PKD1 (solid bars). After 4 days when the cells were confluent and quiescent, the cultures were washed and incubated at 37°C in 2 ml of DMEM/Waymouth’s medium containing either 50 nM vasopressin (VP) or 50 nM ANGII. After 16 h of incubation at 37°C, ³H-labeled thymidine (0.2 µCi/ml, 1 µM) was added and the cultures were incubated for a further 6 h at 37°C. DNA synthesis was assessed by measuring the [³H]thymidine incorporated into acid-precipitable material. Results are expressed as mean cpm/culture x 10^{-3} ± SE (n = 3).

(Lower right), IEC-18 cells were transfected with either non-targeting negative control (open bars) or 75 nM siRNA targeting PKD1 (solid bars) and suspended cells (5 x 10^{5}) were plated onto 35-mm Nunc petri dishes with 2 ml of DMEM containing 1% fetal bovine serum. At day 0 (24 h after plating), the cultures were washed twice with DMEM to remove residual serum and transferred to DMEM/Waymouth (1:1, vol/vol) supplemented with either 50 nM vasopressin (VP) or 50 nM ANGII. Cell number was determined by counting trypsinized cells with a Coulter counter. Cell counts were obtained 24h after the addition of the agonists. (Inset), IEC-18 cells were transfected with either non-targeting negative control (-) or 75 nM siRNA targeting PKD1 (+). After four days when the cells were confluent and quiescent, the cultures were washed and incubated at 37°C in 2 ml of DMEM/Waymouth’s medium containing 50 nM angiotensin (Ang II) for 2 h. The cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with a c-Fos antibody.

**Figure 4. Generation of PKD1 transgenic mice.** Panel A: The FABP promoter (-596 to +21) was fused to the cDNA for PKD (+40 to +2910) and the bovine growth hormone polyadenylation signal sequence, as described in Experimental Procedures. A schematic diagram of the transgene construct produced is shown in panel A. The transgene was excised by digestion with EcoRI and XhoI, gel purified and microinjected into FVB/N mouse oocytes. Panel B: The integrated transgene was detected by PCR as a product of 600bp using genomic tail DNA and specific oligonucleotide primers F12 and BGH (see Materials and Methods for sequences). We verified that PKD protein is overexpressed in epithelial cell lysates of the distal small intestine (ileum) of PKD1 transgenic mice (lines 409 and 417), as shown by Western blotting with PKD C-20 antibodies and as compared with non-transgenic littermate mice (a faint PKD1 band can be seen in NTg). We verified that transgenic PKD1 co-migrated in SDS-PAGE with PKD1 overexpressed in Swiss 3T3 cells, used as a control. Panel C: Histological analysis of ileum of PKD1 transgenic and non-transgenic littermates. Sections (4μm) of paraffin embedded ileal tissue were deparaffinized and stained with a PKD1 antibody purchased from Epitomics (cat# 1986-1). Panel D: Catalytic activity and phosphorylation of PKD extracted from control and transgenic mice. PKD1 was immunoprecipitated from scraped ileal extracts of obtained from transgenic (line 409) and nontransgenic littermate mice. Inset, PKD1 was immunoprecipitated from the lung of transgenic mice. In both cases following elution from the immunocomplexes with the immunizing peptide, PKD1 activity was measured by in vitro kinase assays using syntide-2 as a substrate in the absence (open bars) or in the presence (closed bars) of the lipid activators phosphatidylycerine (PS) and PDBu. Panel E: Transgenic PKD from lines 409 and 417
was phosphorylated at the activation loop residues, Ser\textsuperscript{744} and Ser\textsuperscript{748} and at the autophosphorylation site, Ser\textsuperscript{916}, as shown by immunoblotting of epithelial cell lysates of the distal small intestine (ileum) with the corresponding site-specific antibodies.

**Figure 5. Transgenic PKD1 expression stimulates intestinal cell proliferation.** The proportion of crypt cell DNA synthesis was determined by immunohistochemical detection of BrdU incorporated into the cell nuclei of crypt cells of the ileum. Transgenic (Tg) mice and non-transgenic (NTg) littermates were injected intraperitoneally with BrdU (100\(\mu\)g/g body weight) and sacrificed 3 h after the injection. Tissue fixation, histological sections and routine staining was performed as described in Experimental Procedures. **Panel A:** Representative histological sections of control and PKD1 transgenic mice. **Panels B and C:** The bars represent the percentage of BdUr-labeled crypt cells (mean ±SEM; n=7) in the PKD1 transgenic mice (closed bars) and in the non-transgenic littermates in transgenic line 409 (panel B) and 417 (panel C). **Panel D:** Higher magnification of selected ileal crypts of non-transgenic (NTg) and PKD1 transgenic mice (Tg) illustrating that the BrdU-positive cells are localized to positions corresponding to transit amplifying cells (NTg) and basal columnar stem cells. **Panel E:** The bars represent the percentage of BdUr-labeled columnar basal cells (CBC, mean ±SEM; n=7) in the PKD1 transgenic mice (closed bars) and in the non-transgenic littermates.

**Figure 6. Morphometric analysis of the crypts of the distal region of small intestines of PKD1 transgenic mice and non-transgenic littermates.** Four-micrometer histological sections were cut and stained with hematoxylin/eosin as described under Experimental Procedures. The following morphometric parameters were measured: ileal crypt circumference (in \(\mu\)m and cell number), cell size (crypt height in \(\mu\)m/crypt height in cell number), crypt depth (in \(\mu\)m and cell number) and total number of cells per crypt. This analysis revealed statistical difference (p<0.01) in crypt depth (in \(\mu\)m and cell number) and in the total number of cells per crypt between PKD1 transgenic mice (closed bars) and non-transgenic littermates (open bars). At least 20 well-oriented crypts corresponding to each of 7 transgenic (Tg) and non-transgenic (NTg) mice were analyzed.
Fig. 1

A

Time (min) 0 2.5 30 60 120 180 240

Angiotensin II

B

Time (min) 0 2.5 30 60 120 180 240

Vasopressin

C

N.Targ. PKD1 siRNA PKD2 siRNA

Time (min) 0 2.5 30 120
Fig. 3
**Fig. 4**

**A**
- Diagram showing the molecular structure of FABP Promoter, Protein Kinase D, and BGH PolyA with a line indicating 600 bp.

**B**
- Diagram showing WT, Tg409, and Tg417 with a PCR product (600 bp) and a lane labeled PKD1 and β-Tubulin.

**C**
- Images showing NTg and Tg417 with lung samples.

**D**
- Graph showing Syntide Phosphorylation CPM x 10^4 with bars for NTg and Tg417 with PS/PDB annotations.

**E**
- Images showing NTg, Tg409, and Tg417 with pSer^916, pSer^744, pSer^748, and β-Tubulin annotations.
Fig. 5
Fig. 6
Protein kinase D1 mediates stimulation of DNA synthesis and proliferation in intestinal epithelial IEC-18 cells and in mouse intestinal crypts
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