Protein Kinase D1 mediates Class IIa Histone Deacetylase Phosphorylation and Nuclear Extrusion in Intestinal Epithelial Cells: Role in Mitogenic Signaling

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Running Title: A PKD1/class IIa HDAC axis in intestinal epithelial cells.

The authors declare no conflicts of interest.
ABSTRACT

We examined whether class IIa histone deacetylases (HDACs) play a role in mitogenic signaling mediated by protein kinase D1 (PKD1) in IEC-18 intestinal epithelial cells. Our results show that class IIa HDAC 4, 5 and 7 are prominently expressed in these cells. Simulation with angiotensin II (ANG II), a potent mitogen for IEC-18 cells, induced a striking increase in the phosphorylation of HDAC4 at Ser\(^{246}\) and Ser\(^{632}\), HDAC5 at Ser\(^{259}\) and Ser\(^{498}\) and HDAC7 at Ser\(^{155}\). Treatment with the PKD family inhibitors kb NB 142-70 and CRT0066101 or siRNA-mediated knockdown of PKD1 prevented ANG II-induced phosphorylation of HDAC 4, 5 and 7. A variety of PKD1 activators in IEC-18 cells, including vasopressin, lysophosphatidic acid or phorbol esters, also induced HDAC4, 5 and 7 phosphorylation. Using endogenously and ectopically expressed HDAC5, we show that PKD1-mediated phosphorylation of HDAC5 induces its nuclear extrusion into the cytoplasm. In contrast, HDAC5 with Ser\(^{259}\) and Ser\(^{498}\) mutated to Ala was localized to the nucleus in both unstimulated and stimulated cells. Treatment of IEC-18 cells with specific inhibitors of class IIa HDACs, including MC1568 and TMP269, prevented cell cycle progression, DNA synthesis and proliferation induced in response to GPCR/PKD1 activation. The PKD1/class IIa HDAC axis also functions in intestinal epithelial cell in vivo, since an increase in the phosphorylation of HDAC4/5 and HDAC7 was demonstrated in lysates of cryptal cells from PKD1 transgenic mice as compared with matched non-transgenic littermates. Collectively, our results reveal a PKD1/class IIa HDAC axis in intestinal epithelial cells leading to mitogenic signaling.
INTRODUCTION

The mammalian intestine is covered by a single layer of epithelial cells that is renewed every 4-6 days throughout adult life. This high rate of turnover plays an essential role in the organization, maintenance and restoration of intestinal tissue integrity. The sequential proliferation, differentiation, crypt-villus migration, and death of the epithelial cells of the intestinal mucosa is tightly regulated by a variety of factors (1, 11, 19). The intracellular signal transduction pathways involved, however, are still incompletely understood.

Recent studies with crypt-derived intestinal epithelial cells indicate that protein kinase D1 (PKD1), the founding and best characterized member of the PKD family (40, 41), plays a critical role in mediating migration, proliferation and signal transduction in these cells (34, 44, 52). Accordingly, multiple growth-promoting stimuli rapidly activate PKD1 catalytic activity in intestinal epithelial cells (2, 5, 39, 44, 52) through activation loop phosphorylation (16, 43, 44, 48). Furthermore, transgenic mice that express elevated PKD1 protein in intestinal epithelial cells display a marked increase in DNA-synthesizing cells in their intestinal crypts and a significant increase in the length and total number of cells per crypt (44). Collectively, these results support the notion that PKD1 signaling is a novel element in the pathway leading to proliferation of intestinal epithelial cells in vitro and in vivo. The mechanism(s) downstream of PKD1, however, remain to be identified.

Class IIa histone deacetylases (HDACs), including HADCS 4, 5, 7 and 9, are thought to regulate gene expression by interacting with various transcription factors to repress their transcriptional activity (14, 36) but the precise mechanism of action of these proteins in signal transduction remains incompletely understood (30, 31). Class IIa HDACs are unique among the HDAC family in that they shuttle between nucleus and cytoplasm in response to extracellular signals. Phosphorylation of specific conserved residues in HADCS 4, 5, 7 and 9 leads to their nuclear extrusion and sequestration in the
cytoplasm, thereby facilitating gene expression (14). PKD1 has been identified as one of the upstream kinases that mediate the phosphorylation and subcellular localization of class IIa HDACs in mesenchymal cells (18, 28, 32, 47) thereby promoting cardiac hypertrophy (10, 47), angiogenesis (13, 49), skeletal muscle gene expression (23), T and B cell receptor function (7, 28, 35) and differentiation into the osteogenic lineage (17). In contrast to mesenchymal cells, little is known about the phosphorylation, dynamic localization and function of class IIa HDACs in epithelial cells. In fact, neither phosphorylation-dependent nuclear/cytoplasmic shuttling of endogenous class II HDACs has been demonstrated in intestinal epithelial cells nor any protein kinase that mediates class IIa HDAC phosphorylation has been identified in these cells.

In our search for substrates that mediate PKD1 signaling in intestinal cells, we examined here the hypothesis that PKD1 participates in a signal transduction pathway triggered by G protein-coupled receptors (GPCRs) through direct phosphorylation of class IIa HDACs leading to their nuclear export, cytoplasmic localization and mitogenic signaling. Our results demonstrate that HDAC 4, 5 and 7 are strongly phosphorylated in intestinal epithelial cells in response to GPCR agonists that induce PKD1 activation and mitogenic signaling in these cells. Using endogenously and ectopically expressed HDAC5, we show that PKD1-mediated phosphorylation of HDAC5 induces its nuclear extrusion into the cytoplasm. Structurally unrelated pharmacological inhibitors of class IIa HDACs prevented cell cycle progression and entry into DNA synthesis induced in response to GPCR/PKD1 activation. Our results reveal a PKD1/class IIa HDAC axis in intestinal epithelial cells that mediates mitogenic signaling.
MATERIALS and METHODS

Cell Culture

The non-transformed rat intestinal epithelial IEC-18 cells (37, 38), originated from intestinal crypt cells, were purchased from ATCC. These cells express Gq-coupled receptors for angiotensin II (ANGII) and vasopressin (2-5, 39, 50, 51) and have been extensively used as a model system to examine signal transduction pathways in response to GPCR activation (2-5, 44, 46, 50, 52). Cultures of IEC-18 cells were maintained as described previously (34, 44). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin, and kept at 37°C in a humidified atmosphere containing 10% CO2 and 90% air. Stock cultures were sub-cultured every 3-4 days. For experimental purposes, IEC-18 cells were seeded in 35-mm dishes at a density of 2 × 10^5 cells/dish.

Immunoblotting and detection of HDAC and PKD1 phosphorylation

Serum-starved, confluent intestinal epithelial 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 antibody and a FUJI LAS-4000 Mini Luminescent Image Analyzer.
**Immunofluorescence**

Immunofluorescence of IEC18 cells was performed by fixing the cultures with ice cold 100% methanol followed by permeabilization with 0.2% Triton X-100. After extensive PBS washing, fixed cells were incubated for 2h at 25°C in blocking buffer (PBS -5% FBS and 2% Bovine Serum Albumin) (BB) and then stained at 4°C overnight with a rabbit polyclonal antibody anti-HDAC5 (1:200) diluted in BB. Subsequently, the cells were washed with PBS at 25°C and stained at 25°C for 60 min with Alexafluor 546 - conjugated goat-anti rabbit diluted in BB (1:100) and washed again with PBS. Nuclei were stained using Hoechst 33342 (1:10,000). The samples were imaged with an epifluorescence Zeiss Axioskop and a Zeiss water objective (Achroplan 40/.75W Carl Zeiss, Inc.). Images were captured as uncompressed 24-bit TIFF files with a cooled (-12°C) single CCD color digital camera (Pursuit, Diagostic Instruments) driven by SPOT version 4.7 software. Alexafluor 546 signals were observed with a HI Q filter set for Rhodamine/TRITC (Chroma Technology). The selected cells displayed in the appropriate figures were representative of 90% of the population.

**Knockdown of PKD1 levels via siRNA transfection**

The pooled siRNA duplexes were purchased from 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 three different duplexes (oligo1, CUCUCUUCGUUCAUAUAAt; oligo2, GUGAGCAUUUCCGUUUCAAtt; oligo3, GAAGCCAUGAUCUUUAUCAtt). 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 Stealth siRNA negative control (Invitrogen,
Carlsbad, CA). Four days after transfection, cells were used for experiments and subsequent Western blot analysis.

**Cell transfection**

IEC-18 cells were transfected with the plasmid containing a cDNA encoding an epitope (FLAG) tagged-HDAC5 wild type or an identical construct in which Ser\(^{259}\) and Ser\(^{498}\) were mutated to non-phosphorylatable Ala from Addgene (cat # 13822 and 32216 respectively) by using Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. Analysis of the cells transiently transfected were performed 48 h after transfection.

**Immunoprecipitation of HDAC 4, 5 and 7.**

Confluent IEC-18 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, 10 mM sodium fluoride, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (Pefabloc) and 1% Triton X-100. The HDACs were immunoprecipitated from the cell extracts with antibodies from Cell Signaling Technology. The immune complexes were recovered using protein-A coupled to agarose.

**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 ANG II and increasing concentrations of the specific class IIa HDAC inhibitors MC1568 and TMP269 as described in the figure legends. After 18 h of incubation at 37 °C, \([^{3}\text{H}]\)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\(_3\), 0.1M NaOH. The acid-insoluble radioactivity was determined by scintillation counting in 6 ml of Beckman Readysafe.
Flow cytometric analysis

The proportion of cells in the G0/G1, S, G2, and M phases of the cell cycle was determined by flow cytometric analysis. Cells were seeded at a density of $1 \times 10^5$ cells in 35-mm dishes in DMEM containing 10% FBS for 4 days. The cells were then washed two times with DMEM and incubated with DMEM containing various additions as described in the legend to Fig. 5 for 6 h before the addition of 1 μM colchicine and incubation for another 24 h. After the indicated treatment, cells were harvested by trypsinization, washed in phosphate buffered saline (PBS), and resuspended in a final concentration of $1 \times 10^6$ cells/ml in hypotonic propidium iodide (PI) solution containing 0.1% sodium citrate, 0.3% Trixon-X 100, 0.01% PI, 0.002% Ribonuclease A. Cells were incubated in 4°C for 30 min before acquisition on the flow cytometer (Becton-Dickinson) using CELLQuest. One hundred thousand cells were collected for each sample. Excitation occurred at 488 nm and data were collected in the channel of FL2 and analyzed using FCS ExpressV3.

Class IIa HDAC phosphorylation in intestinal epithelial cells in vivo.

To assess the effect of PKD1 on class IIa HDAC phosphorylation in vivo, we used transgenic mice that express elevated PKD1 protein in the ileal epithelium and control non-transgenic littermates. The generation of PKD1 transgenic mice was described elsewhere (44). To perform anatomical dissection and tissue collection mice were euthanized in a CO2 chamber. Overexpression of PKD1 in the ileum was verified using epithelial cells isolated sequentially along the crypt-villus axis by timed incubations in EDTA-PBS solutions. To measure PKD1 expression and HDAC phosphorylation, lysates of intestinal cells isolated from gender- and age-matched mice were subjected to immunoblotting, as described above. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee of the University of California, Los Angeles (Protocol Number: 2001-142–23).
Materials

DMEM was obtained from Invitrogen (Carlsbad, CA). Angiotensin II, vasopressin and lysophosphatic acid (LPA) were obtained from Sigma Chemical (St. Louis, MO). kb NB 142-70 was obtained from R&D Systems (Minneapolis, MN) and CRT0066101 was obtained from Cancer Research Technology Discovery Laboratories (London, UK). The specific class IIa HDAC inhibitor MC1568 was purchased from Selleck Chemicals (Houston, TX) and TMP269 from Xcessbio (San Diego, CA). All antibodies, including the antibody that detects the phosphorylated state of HDAC 4 at Ser$^{246}$, HDAC5 at Ser$^{259}$ and HDAC7 at Ser$^{155}$ and the antibody that recognizes the phosphorylated state HDAC4 at Ser$^{632}$, HDAC5 at Ser$^{498}$ and HDAC7 at Ser$^{486}$ were purchased from Cell Signaling Technology (Danvers, MA). All other reagents were of the highest grade available.

RESULTS

Expression and GPCR-induced phosphorylation of class II HDACs in IEC-18 cells.

In order to determine whether class IIa HDACs are expressed in intestinal epithelial IEC-18 cells, lysates of these cells were analyzed by Western blotting using antibodies that recognize class IIa HDACs 4, 5, 7 and 9. As shown in Fig 1 A, robust immunoreactive signals for HDAC 4, 5 and 7 were detected in IEC-18 extracts whereas only a faint band was seen with the antibody directed against HDAC 9 (results not shown). Immunoprecipitation with specific anti-HDAC antibodies followed by Western blotting verified that IEC-18 cells express multiple class IIa HDACs, i.e. HDACs 4, 5 and 7 (Fig. 1 B).

Next, we determined whether stimulation of IEC-18 cells with a GPCR agonist enhances the phosphorylation of class II HDACs in these cells. We used an antibody that detects the phosphorylated state of HDAC 4 at Ser$^{246}$, HDAC5 at Ser$^{259}$ and HDAC7 at Ser$^{155}$ and a second antibody that recognizes
the phosphorylated state HDAC4 at Ser$^{632}$, HDAC5 at Ser$^{498}$ and HDAC7 at Ser$^{486}$ (Fig. 1 C). Simulation of IEC-18 cells with the Gq-coupled receptor agonist angiotensin II (ANG II), a potent mitogen for IEC-18 cells (3, 4, 44), induced striking phosphorylation of class IIa HDACs in these cells, as detected with both antibodies in whole lysates of these cells (Fig. 1 C). The first antibody revealed two bands: the upper band corresponded to phosphorylated HDAC4 at Ser$^{246}$ and/or HDAC5 at Ser$^{259}$ whereas the lower band corresponded to phosphorylated HDAC7 at Ser$^{155}$. Western blotting with the second phospho-specific HDAC antibody showed phosphorylation of HDAC4 at Ser$^{632}$ and/or HDAC5 at Ser$^{498}$ (Fig. 1 C).

To confirm the identity of the phosphorylated bands detected in the cell lysates, endogenous HDAC 4, 5 and 7 were recovered from IEC-18 cell extracts by immunoprecipitation and the resulting immunoprecipitates were analyzed by Western blotting with the phospho-specific HDAC antibodies. As shown in Fig. 1 C, cell stimulation with ANG II induced a striking increase in the phosphorylation of HDAC4 at Ser$^{246}$ and Ser$^{632}$, HDAC5 at Ser$^{259}$ and Ser$^{498}$ and HDAC7 at Ser$^{155}$. These results demonstrated, for the first time, stimulus-dependent robust phosphorylation of class IIa HDACs in intestinal epithelial cells.

The PKD family inhibitors kb NB 142-70 and CRT0066101 or knockdown of PKD1 prevented class IIa HDAC phosphorylation in IEC-18 cells.

In order to determine the role of PKD family in mediating ANG II-induced class IIa HDAC phosphorylation in IEC-18 cells, we used the recently identified preferential PKD family inhibitors kb NB 142-70 and CRT0066101 (15, 22) which act as potent PKD1 inhibitors in intact IEC-18 cells (34, 52). Cultures of IEC-18 cells were treated with increasing concentrations of kb NB 142-70 (Fig. 2, A) or CRT0066101 (Fig. 2, B) for 1 h and then stimulated with ANG II. Prior exposure to either kb NB 142-70 or CRT0066101 prevented ANG II-induced phosphorylation of HDAC4 at Ser$^{246}$ and Ser$^{632}$, HDAC5 at
Ser^{259} and Ser^{498} and HDAC7 at Ser^{155} in a concentration-dependent manner. The concentrations of kb NB 142-70 and CRT0066101 that blunted ANG II-induced phosphorylation of HDAC were virtually identical to those required to suppress PKD1 activity within IEC-18 cells, as shown in our previous studies (34, 52) by monitoring autophosphorylation at Ser^{916} in the C-terminal region (29) and Ser^{748} in the activation loop of the kinase catalytic domain (16, 43).

To prove that PKD1 mediates phosphorylation of class IIa HDACs in response to GPCR activation in IEC-18 cells, we determined whether knockdown of PKD1 expression prevents ANG II-induced HDAC phosphorylation in these cells. The PKD1 protein level in IEC-18 cells transfected with siRNA targeting PKD1 was dramatically reduced (>90%) compared with cells transfected with nontargeted negative control duplex (Fig. 2C). In contrast, the level of PKD2 protein, the lower band of the doublet detected with the PKD1 C-20 antibody (44), was not affected. PKD1 knockdown strikingly prevented class IIa HDAC phosphorylation in response to ANG II stimulation in IEC-18 cells (Fig. 2C). Collectively, the results with PKD family inhibitors, multiple PKD1 activators and siRNA-mediated knockdown of PKD1 indicate that this member of the PKD family is the predominant PKD isoform mediating class IIa HDAC phosphorylation in response to GPCR agonists in intestinal epithelial cells.

It was possible that phosphorylation of HDAC4, 5 and 7 could be mediated by different protein kinases at different times after GPCR stimulation. Consequently, we examined the time-course of ANG II-induced class IIa phosphorylation in IEC-18 preincubated with or without kb NB 142-70. As shown in Fig. 3A, striking phosphorylation of HDAC4 at Ser^{246} and Ser^{632}, HDAC5 at Ser^{259} and Ser^{498} and HDAC7 at Ser^{155} was already nearly maximal after 10 min of ANG II stimulation and persisted at for at least 4 h. Prior exposure to the PKD family inhibitor kb NB 142-70 drastically reduced class IIa HDAC phosphorylation at all times examined.
We next determined whether stimulation of IEC-18 cells with other GPCR agonists that induce PKD1 activation in these cells also elicit class IIa HDAC phosphorylation through PKD1. As shown in Fig. 3B, stimulation of IEC-18 cells with vasopressin (5, 39, 44), LPA (2) or PDBu enhanced phosphorylation of HDAC4 at Ser\(^{246}\) and Ser\(^{632}\), HDAC5 at Ser\(^{259}\) and Ser\(^{498}\) and HDAC7 at Ser\(^{155}\). In each case, prior exposure to kb NB 142-70 markedly attenuated the phosphorylation of these HDACs in IEC-18 cells. These results imply that PKD1 mediates class IIa HDAC phosphorylation in response to multiple GPCR agonists and phorbol esters in intestinal epithelial IEC-18 cells.

**PKD1 regulates class IIa HDAC nucleo-cytoplasmic shuttling in response to GPCR activation in IEC-18 cells**

To determine the functional significance of class IIa HDAC phosphorylation in GPCR-mediated signaling in epithelial cells, we examined the effect of ANG II on endogenous HDAC5 nucleo-cytoplasmic shuttling. Immunofluorescence analysis showed that endogenous HDAC5 localizes predominantly to the nucleus in unstimulated IEC-18 cells (Fig. 4). Stimulation with ANG II for 1h induced a striking intracellular redistribution of HDAC5, from the nucleus to the cytosol (Fig. 4) that persisted for at least 4 h (results not shown). Prior exposure to kb NB 142-70 did not cause any detectable change in the cellular distribution of HDAC5 in unstimulated IEC-18 cells but prevented its nuclear extrusion and cytoplasmic localization induced by ANG II (Fig. 4). These results indicate that HDAC5, a typical class IIa HDAC, shuttles between nucleus and cytoplasm in intestinal epithelial cells in response to GPCR activation and that PKD1 plays a critical role in regulating this process.

We next determined whether the nuclear extrusion of HDAC5 is mediated by phosphorylation of the residues strongly phosphorylated in response to GPCR/PKD1 activation. Cultures of IEC-18 cells were transfected with epitope (FLAG)-tagged HDAC5 or an identical construct in which Ser\(^{259}\) and Ser\(^{498}\) were mutated to non-phosphorylatable Ala. After 48h, the cells were treated with or without kb NB 142-
70 and subsequently challenged with ANG II. As shown in Fig. 5, FLAG-HDAC5 is present in the nucleus of unstimulated cells and strikingly translocates to the cytosol in response to ANG II stimulation. Treatment with kb NB 142-70 prevented the nuclear extrusion of FLAG-HDAC5, in line with the results shown with endogenous HDAC5 (Fig. 4). In contrast, FLAG-HDAC5 with Ser$^{259}$ and Ser$^{498}$ mutated to Ala were localized to the nucleus in both unstimulated and GPCR-stimulated cells (Fig. 5), indicating that the phosphorylation of these residues plays a critical role in mediating nuclear/cytoplasmic HDAC5 shuttling in intestinal epithelial cells.

**Role of class IIa HDAC activity in GPCR-induced mitogenesis in IEC-18 cells**

Previous results demonstrated that PKD1 activation plays a key role in mediating GPCR-induced cell proliferation in intestinal epithelial cells (44) but the mechanism(s) downstream of PKD1 remain to be identified. Having established that phosphorylation, nuclear export and cytoplasmic localization of class IIa HDACs through PKD1 is an early event in GPCR-induced signaling in intestinal epithelial cells, we next determined whether class IIa HDAC activity plays a role in promoting the mitogenic response induced through the GPCR/PKD1 pathway. Cultures of IEC-18 cells in serum-free medium were stimulated with ANG II in the absence or presence of increasing concentrations of the specific class IIa HDAC inhibitor MC1568 (8, 25, 33) and DNA synthesis was assessed by measuring $[^{3}H]$thymidine incorporation into acid-precipitable material. As shown in Fig. 6 A, addition of MC1568 prevented ANG II-induced DNA synthesis in IEC-18 cells in a dose-dependent manner. Half-maximal inhibitory effect was elicited at ~3 μM. In order to substantiate the results obtained with MC1568, we also tested TMP269, a recently developed specific inhibitor of class IIa HDAC which contains a trifluoromethylloxadiazolyl moiety and is therefore structurally unrelated to MC1568 (24). Exposure of IEC-18 cells to increasing concentrations of TMP269 potently inhibited $[^{3}H]$thymidine incorporation induced by ANG II in these cells. Half-maximal inhibitory effect was elicited at ~1.5 μM (Fig. 6 B). These results indicated that class
IIa HDAC catalytic domain activity is necessary for mitogenic signaling induced via GPCR/PKD1 in intestinal epithelial cells.

To substantiate that the inhibitory effects elicited by exposure to either MC1568 or TMP269 on $[^3]H$thymidine incorporation reflects a blockade in cell cycle progression rather than an alteration(s) in $[^3]H$thymidine uptake (e.g. transport and/or phosphorylation), we used flow cytometric analysis to determine the proportion of cells in the various phases ($G_0/G_1$, $S$, and $G_2/M$) of the cell cycle. Confluent and serum-starved cultures of IEC-18 cells were stimulated with ANG II either in the absence or presence of 5 μM MC1568 or 4 μM TMP269. Cells that traversed the cell cycle were accumulated in $G_2/M$ by addition of colchicine. As shown in Fig. 6C, stimulation of IEC-18 cells with ANG II induced a striking shift from $G_0/G_1$ to $G_2/M$, an effect completely prevented by cell exposure to either MC1568 or TMP269. Furthermore, the inhibitors of class IIa HDACs also abolished the increase in cell number induced by ANG II in IEC-18 cells (Fig. 6, D). Collectively, the results demonstrate, for the first time, that pharmacological inhibition of class IIa HDAC activity completely prevented GPCR/PKD1-induced progression of the cell cycle, DNA synthesis and proliferation in IEC-18 cells.

As controls, we verified that cell exposure to either MC1568 (1-5 μM) or TMP269 (1-5 μM) neither prevented PKD1 activation in response to ANG II in IEC-18 cells, scored by autophosphorylation at Ser$^{916}$, nor PKD1-mediated phosphorylation of HDAC 4 at Ser$^{246}$ and HDAC5 at Ser$^{259}$ in these cells (Fig. 7, A). Furthermore, treatment with either MC1568 or TMP269 did not interfere with GPCR/PKD1-induced nuclear extrusion and cytoplasmic localization of endogenous HDAC5 in IEC-18 cells (Fig. 7, B). These results imply that class IIa HDACs catalytic pocket plays a role in mitogenic signaling when these proteins are localized in the cytoplasm of intestinal epithelial cells.

**Over-expression of PKD1 enhances class II HDAC phosphorylation in intestinal epithelial cells “in vivo”**.
Our preceding results indicating that PKD1 is required for class II HDAC phosphorylation and redistribution in IEC-18 cells, prompted us to determine whether PKD1 promotes HDAC phosphorylation of intestinal epithelial cells \textit{in vivo}. In order to examine this possibility, we used transgenic mice that express elevated PKD1 protein in the small intestine epithelium and display a marked increase in DNA-synthesizing cells in their intestinal crypts and a significant increase in the length and total number of cells per crypt (44). As shown in Fig. 8 A, over-expression of PKD1 protein in the ileum was verified by Western blot analysis of total PKD1 in lysates of epithelial cells isolated sequentially along the crypt-to-villus axis by timed incubations in EDTA-PBS solutions (9, 27). Using these lysates, we also demonstrated that HDAC 5 is prominently expressed in fractions 6 and 8 of epithelial cells, corresponding to the proliferative compartment of the crypt, as shown by the detection of PCNA in these fractions. Furthermore, the results, shown in Fig. 8 B demonstrated that class IIa HDAC phosphorylation was markedly increased in the epithelial cells isolated from PKD1 transgenic mice as compared with its non-transgenic littermates. The results support the notion that PKD1 promotes the phosphorylation of class IIa HDACs in intestinal epithelial cells \textit{in vivo}.

**DISCUSSION**

PKD1 has been identified as one of the upstream kinases that mediate the phosphorylation and subcellular localization of class IIa HDACs in \textit{mesenchymal} cells (18, 28, 32, 47) thereby promoting cardiac hypertrophy (10, 47), angiogenesis (13, 49), skeletal muscle gene expression (23), T and B cell receptor function (7, 28, 35) and differentiation into the osteogenic lineage (17). In contrast to mesenchymal cells, little is known about the phosphorylation, dynamic localization and function of class IIa HDACs in epithelial cells. Consequently, the experiments presented here were designed to determine whether PKD1 regulates the phosphorylation and redistribution of class IIa HDACs in intestinal epithelial cells and explore the role of class IIa HDACs in mitogenic signaling induced through the GPCR/PKD1
pathway in these cells. Using IEC-18 cells as a model system of intestinal epithelial crypt cells, we produced here several lines of evidence indicating that PKD1 is major upstream kinase that mediates class IIa HDAC phosphorylation and nuclear extrusion: 1) Stimulation of IEC-18 cells with ANG II, a potent mitogen for these cells, induced a striking increase in the phosphorylation of HDAC4 at Ser\textsuperscript{246} and Ser\textsuperscript{632}, HDAC5 at Ser\textsuperscript{259} and Ser\textsuperscript{498} and HDAC7 at Ser\textsuperscript{155}. These results demonstrated, for the first time, robust class IIa HDAC phosphorylation in response to GPCR activation in intestinal epithelial cells; 2) Treatment of IEC-18 cells with the structurally unrelated PKD family inhibitors kb NB 142-70 and CRT0066101 markedly reduced ANG II-induced phosphorylation of HDAC 4, 5 and 7 in a dose-dependent manner; 3) A variety of PKD1 activators in IEC-18 cells, including vasopressin (5, 39), LPA (50) and phorbol esters (2), also induced HDAC4, 5 and 7 phosphorylation; 3) siRNA-mediated selective knockdown of PKD1 protein expression prevented ANG II-induced class IIa HDAC phosphorylation; 4) Immunofluorescence analysis showed that stimulation with ANG II induced a striking intracellular redistribution of endogenous HDAC5, from the nucleus to the cytosol; 5) Using HDAC5 as a model of class IIa HDAC, we demonstrate that residues targeted by PKD1 play a critical role in mediating nuclear extrusion and cytoplasmic localization in response to GPCR activation; 6) The PKD1/class IIa HDAC axis also occurs in intestinal epithelial cell \textit{in vivo}, since an increase in the phosphorylation of HDAC4/5 and HDAC7 was demonstrated in lysates of cryptal cells from mice that express elevated PKD1 protein in intestinal epithelial cells. Collectively, these results demonstrate, for the first time, that PKD1 mediates endogenous class IIa phosphorylation, nuclear extrusion and cytoplasmic localization in response to GPCR activation in intestinal epithelial cells.

ANG II induces DNA synthesis and proliferation in IEC-18 cells (3, 4, 44) via its cognate Gq-coupled receptor endogenously expressed by these intestinal epithelial cells (3, 51). Recent studies
revealed that PKD1 activation plays a critical role in mediating ANG II-induced proliferative responses in these cells (44). PKD1 is also rapidly activated by mitogenic stimuli and mediates growth-promoting signaling in Swiss 3T3 fibroblasts (43, 45, 53, 54) and human pancreatic cancer cells (12, 15, 20) but the mechanism(s) downstream of PKD1 remains incompletely understood in all model systems. In our search for substrates that mediate PKD1 signaling in intestinal cells, we tested the hypothesis that PKD1-mediated class IIa HDAC phosphorylation and intracellular relocalization participates in a signal transduction pathway triggered by GPCRs leading to mitogenesis. To explore this hypothesis we examined whether pharmacological inhibition of class IIa HDAC activity, using MC1568 (8, 25, 26, 33) and TMP269 (24), interferes with ANG II mitogenic signaling. We show here that exposure of IEC-18 cells to either MC1568 or the more recently identified class IIa HDAC inhibitor TMP269 prevented the stimulation of DNA synthesis induced by the GPCR agonist ANG II. More evidence for a functional role of class IIa HDAC activity in the regulation of the proliferative response was derived from cell cycle analysis by flow cytometry demonstrating that either MC1568 or TMP269 block cell cycle progression in G0/G1. The results imply that class IIa HDACs are critical players in GPCR/PKD1 mitogenic signaling in intestinal epithelial cells.

In theory, class IIa HDACs could contribute to mitogenic signaling in IEC-18 cells through at least two mechanisms. Since GPCR/PKD1 signaling induces potent nuclear extrusion of class IIa HDACs in these cells, a first mechanism could be the removal of their transcriptional repressive effects in the nucleus mediated by the scaffolding function of the N-terminal region of these proteins. If this was the only or predominant mechanism, pharmacological inhibition of class IIa HDACs catalytic activity should not interfere with mitogenic signaling. On the other hand, the results presented here with recently developed specific inhibitors, support an unexpected second mechanism in which class IIa HDACs function in mitogenic signaling via their HDAC catalytic domain in the cytoplasm (21, 42). In this
context, characterization of lysine acetylation sites revealed that many proteins involved in cytoskeleton organization, cell cycle and signal transduction are acetylated and reside in the cytoplasm (6). Because the deacetylase activity of class IIa HDACs is ~1000 lower than class I HDACs for acetylated histones (21, 42) it is conceivable that class IIa HDACs act on cytoplasmic substrates that remain, as yet, to be identified. Alternatively, the catalytic domain may mediate binding to acetyllysine residues of proteins to promote protein–protein interactions (24), thereby regulating the assembly of multiprotein signaling complexes in the cytoplasm (6). Our results imply that the occupation of the catalytic domain of class IIa HDACs in the cytoplasm by either MC1568 or TMP269 blocks the stimulation of cell cycle progression in intestinal epithelial cell proliferation. Our findings identify, for the first time, a novel function in mitogenic signaling for cytoplasmic class IIa HDACs in intestinal epithelial cells.

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55. FIGURE LEGENDS

56. Figure 1. Expression and phosphorylation of class IIa HDACs in IEC-18 cells. A. Confluent cultures of IEC-18 cells were lysed with 2×SDS–PAGE sample buffer. The lysates were analyzed by SDS-PAGE and immunoblotting with antibodies that detect HDAC4, HDAC5 and HDAC7. B Confluent cultures of IEC-18 cells were lysed with lysis buffer A and the extracts were immunoprecipitated with specific antibodies to HDAC4, HDAC5 and HDAC7. Immunoprecipitates were analyzed by immunoblotting using antibodies that detect total HDAC4, HDAC5 and HDAC7, as indicated. C, Confluent cultures of IEC-18 cells were incubated without (-) or with 50 nM angiotensin II (ANG II) for 1 h. The cells were then lysed and the...
immunoprecipitates were analyzed by immunoblotting with an antibody that detects the phosphorylated state of HDAC 4 at Ser$^{246}$, HDAC5 at Ser$^{259}$ and HDAC7 at Ser$^{155}$ or an antibody that recognizes the phosphorylated state HDAC4 at Ser$^{632}$ and HDAC5 at Ser$^{498}$, as indicated.

Figure 2. PKD family inhibition by kb NB 142-70 or CRT006101 or siRNA knockdown of PKD1 prevents HDAC 4, 5 and 7 phosphorylation in IEC-18 cells. A and B, Confluent cultures of IEC-18 cells were incubated in the absence (-) or presence of increasing concentrations kbNB 142-70 or CRT006101 for 1 h prior to stimulation without (-) or with 50 nM angiotensin II (ANG II) for 1 h, as indicated. All cultures were then lysed with 2×SDS–PAGE sample buffer. C. Cultures of IEC-18 cells were transfected with non-targeting siRNA (NTarg) or with siRNAs targeting PKD1. Other cultures were not subjected to transfection (Cont). Then, the cultures were stimulated with 50 nM angiotensin II (ANG II) for 1 h and lysed with 2×SDS–PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotting with an antibody that detects the phosphorylated state of HDAC 4 at Ser$^{246}$, HDAC5 at Ser$^{259}$ and HDAC7 at Ser$^{155}$ or an antibody that recognizes the phosphorylated state HDAC4 at Ser$^{632}$ and HDAC5 at Ser$^{498}$. Antibodies directed against total HDAC5 and HDAC 7 were used to verify equal gel loading. In panel C, total PKD1 and PKD2 were detected with the antibody PKD-C20 to evaluate siRNA-mediated knockdown of PKD1 expression. Similar results were obtained in at least 3 independent experiments in each case.

Figure 3. PKD1 inhibition by kb NB 142-70 prevents HDAC 4,5 and 7 phosphorylation in IEC-18 cells stimulated with either ANG II for various times or by vasopressin, LPA or...
Confluent cultures of IEC-18 cells were incubated in the absence (-) or presence 3.5 μM kb NB 142-70 (kb) for 1 h prior to stimulation of the cells with 50 nM angiotensin II (ANG II) for various times, as indicated. Confluent cultures of IEC-18 cells were incubated in the absence (-) or presence 3.5 μM kb NB 142-70 (kb) for 1 h prior to stimulation of the cells with 50 nM angiotensin II (ANG II), 50 nM vasopressin (VP), 10 μM LPA or 100 ng/ml PDBu. All samples were analyzed for HDAC 4, 5 and 7 phosphorylations with an antibody that detects the phosphorylated state of HDAC 4 at Ser$^{246}$, HDAC5 at Ser$^{259}$ and HDAC7 at Ser$^{155}$ or a different antibody that recognizes the phosphorylated state HDAC4 at Ser$^{632}$ and HDAC5 at Ser$^{498}$. Antibodies directed against total HDAC5 and HDAC 7 were used to verify equal gel loading. Similar results were obtained in at least 3 independent experiments in each case.

**Figure 4. PKD1 regulates endogenous HDAC5 nucleo-cytoplasmic shuttling in response to ANGII stimulation in IEC-18 cells.** Confluent cultures of IEC-18 cells were incubated in the absence (-) or presence of 3.5 μM kbNB 142-70 (kb) for 1 h prior to stimulation of the cells without (-) or with 50 nM angiotensin II (ANG II) for 1 h. The cultures were then washed, fixed in ice cold methanol and stained with an antibody that detects HDAC5 and with Hoechst 33342 stain to visualize the cell nuclei, as described in “Materials and Methods”.

**Figure 5. Mutations of Ser$^{259}$ and Ser$^{498}$ to Ala in HDAC5 prevents its nuclear extrusion.** IEC-18 cells were transiently transfected with a plasmid encoding FLAG tagged-HDAC5 or FLAG tagged-HDAC5S259A and S498A. The cultures were incubated in the absence (-) or presence of 3.5 μM kb NB 142-70 (kb) for 1 h prior to stimulation with 50 nM angiotensin II (ANG II). The cultures were then then washed and fixed with 4% paraformaldehyde and stained
with an antibody that detects the FLAG tag and Hoechst 33342 stain to visualize the nuclei, as described in “Materials and Methods”.

**Figure 6. Class IIa HDAC catalytic domain activity is necessary for mitogenic signaling induced by ANGII in intestinal epithelial cells.** Confluent cultures of IEC-18 cells were incubated with increasing concentrations of either MC1968 (A) or TMP269 (B) for 1 h prior to stimulation of the cells with 50 nM ANG II. 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 = 6).

C. Confluent IEC-18 cells were incubated either in the absence (-) or presence of MC1568 (5 µM) or TMP269 (3 µM) and 50 nM ANG II for 6 h before the addition of 1 µM colchicine and incubation for another 24 h. The colchicine was added to arrest cells that progressed through the cell cycle at G2/M. The proportion of cells in G0/G1 and G2/M, determined by flow cytometric analysis, was 71 ± 0.7 and 16 ± 0.4 in the control cells, 38 ± 0.8 and 54 ± 0.6 in the cells stimulated with ANG II, 92 ± 0.1 and 5 ± 0.3 in the cells stimulated with ANG II but treated with MC1568 and 83 ± 0.8 and 11 ± 0.2 in the cells stimulated with ANG II but treated with TMP269. The proportion of cells in the G0/G1 and G2/M phases of the cell cycle that were treated with MC1568 or TMP269 but without ANG II were virtually identical to those treated with ANG II. The shift from G0/G1 to G2/M induced by 50 nM ANG II was equivalent to that produced by addition of 10% FBS to parallel cultures, used as a positive control (result not shown). D. Suspended IEC-18 cells (5 × 10⁴) 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 in the absence
(open bar) or presence of increasing concentrations of either MC1568 or TMP269, as indicated.
Cultures represented by the closed bars also received 50 nM ANGII. Cell number was
determined by counting trypsinized cells with a Coulter counter. Cell counts were obtained 48 h
after the addition of the agonists. * p<0.05

Figure 7. Class IIa HDAC inhibition by either MC1568 or TMP269 does not prevent PKD1
activation, HDAC 4 and 5 phosphorylation and HDAC5 nuclear extrusion in IEC-18 cells
stimulated with ANG II. A, Confluent cultures of IEC-18 cells were incubated in the absence
(-) or presence MC1568 (5 μM) or TMP269 (4 μM) for 1 h prior to stimulation of the cells
without (0) or with 50 nM angiotensin II (ANG II) for either 10 min or 60 min, as indicated.
Samples were analyzed for HDAC 4/5 phosphorylation with an antibody that detect the
phosphorylated state of HDAC 4 at Ser246 and HDAC5 at Ser259. Similar results were obtained
in at least 3 independent experiments in each case. B, Confluent cultures of IEC-18 cells were
incubated in the absence (-) or presence of 5 μM MC1568 or 3 μM TMP269 for 1 h prior to
stimulation of the cells without (0) or with 50 nM angiotensin II (ANG II) for 1 h. The cultures
were then washed, fixed in ice cold methanol and stained with an antibody that detects HDAC5
and with Hoechst stain to visualize the cell nuclei, as described in “Materials and Methods”.

Figure 8. Over-expression of PKD1 enhances class II HDAC phosphorylation in intestinal
epithelial cells “in vivo”. A. PKD1 is overexpressed along the crypt/villus axis in PKD1
transgenic mice (Tg) as compared with non-transgenic littermates (NTg). Epithelial cells from
the ileum of Tg and NTg mice were isolated sequentially, collected as fractions, (fractions 2, 4, 6
and 8 are shown) along the crypt-to-villus axis by timed incubations in EDTA-PBS solution. Lysates of these cells were analyzed by Western blot analysis for total PKD1, HDAC5 and the proliferation marker PCNA (Proliferating cell nuclear antigen). Sequential elution along the crypt-to-villus axis was verified by the gradient of PCNA expression. B. Eluted cells from 2 separate fraction 6 (I and II) preparations were lysed and the extracts used for analysis by SDS-PAGE and immunoblotting with antibodies that detect total PKD and PKD1 autophosphorylated on Ser\textsuperscript{916}. The lysates were also analyzed with the antibody that recognizes the phosphorylated state of HDAC4 at Ser\textsuperscript{632}, HDAC5 at Ser\textsuperscript{498} and HDAC7 at Ser\textsuperscript{486} or an antibody that recognizes the phosphorylated state HDAC 4 at Ser\textsuperscript{246}, HDAC5 at Ser\textsuperscript{259} and HDAC7 at Ser\textsuperscript{155} and with antibodies directed against total HDAC 4, HDAC5 and HDAC 7. Results are shown for 2 PKD1 transgenic mice and 2 nontransgenic littermates. Bars: represent the level of phosphorylation of HDAC4 at Ser\textsuperscript{632}, and HDAC5 at Ser\textsuperscript{498} (means ± SE; n=5) and HDAC 4 at Ser\textsuperscript{246}, HDAC5 at Ser\textsuperscript{259} and HDAC7 at Ser\textsuperscript{155} (means ± SE; n=6). * p<0.05
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
**Fig. 6**

A. 

[Graph showing Thymidine Incorporation (% of maximum) vs. MC1568 concentration (µM) and TMP269 concentration (µM)].

B. 

[Graph showing Thymidine Incorporation (% of maximum) vs. MC1568 concentration (µM) and TMP269 concentration (µM)].

C. 

[Graphs showing cell cycle distribution (Go/G1, S, G2+M) for ANGII, ANGII + MC1568, and ANGII + TMP269].

D. 

[Bar graphs showing cell number (x 10^-4) for MC1568 and TMP269 concentrations].
Fig. 7
Fig. 8