Published in final edited form as:

J Cell Physiol. 2010 May ; 223(2): 309–316. doi:10.1002/jcp.22036.

Induced Overexpression of Protein Kinase D1 Stimulates Mitogenic Signaling in Human Pancreatic Carcinoma PANC-1 Cells[#]

Krisztina Kisfalvi¹, Cliff Hurd¹, Sushovan Guha², and Enrique Rozengurt^{1,¶}

¹ Department of Medicine, Division of Digestive Diseases; CURE: Digestive Diseases Research Center; David Geffen School of Medicine, and Molecular Biology Institute, University of California, Los Angeles, CA 90095

² Department of Gastroenterology, Hepatology and Nutrition, The University of Texas M. D. Anderson Cancer Center, Houston, 77030, USA

Abstract

Neurotensin (NT) stimulates protein kinase D1 (PKD1), extracellular signal regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and DNA synthesis in the human pancreatic adenocarcinoma cell line PANC-1. To determine the effect of PKD1 overexpression on these biological responses, we generated inducible stable PANC-1 clones that express wild-type (WT) or kinase-dead (K618N) forms of PKD1 in response to the ecdysone analog ponasterone-A (PonA). NT potently stimulated c-Jun Ser⁶³ phosphorylation in both wild type and clonal derivatives of PANC-1 cells. PonA-induced expression of WT, but not K618N PKD1, rapidly blocked NT-mediated c-Jun Ser⁶³ phosphorylation either at the level of or upstream of MKK4, a dual-specificity kinase that leads to JNK activation. This is the first demonstration that PKD1 suppresses NT-induced JNK/cJun activation in PANC-1 cells. In contrast, PKD1 overexpression markedly increased the duration of NT-induced ERK activation in these cells. The reciprocal influence of PKD1 signaling on pro-mitogenic ERK and pro-apoptotic JNK/c-Jun pathways prompted us to examine whether PKD1 overexpression promotes DNA synthesis and proliferation of PANC-1 cells. Our results show that PKD1 overexpression increased DNA synthesis and cell numbers of PANC-1 cells cultured in regular dishes or in polyhydroxyethylmethacrylate [Poly-(HEMA)]-coated dishes to eliminate cell adhesion (anchorage-independent growth). Furthermore, PKD1 overexpression markedly enhanced DNA synthesis induced by NT (1–10 nM). These results indicate that PKD1 mediates mitogenic signaling in PANC-1 and suggests that this enzyme could be a novel target for the development of therapeutic drugs that restrict the proliferation of these cells.

Keywords

neurotensin; c-Jun-N-terminal kinase; ERK; c-jun; G protein-coupled receptor

[#]This work was funded partly by the Ronald S. Hirshberg Memorial Foundation for Pancreatic Cancer Research and by NIH Grants R21CA137292, RO1DK56930, RO1DK55003 and P30DK41301 (to ER).

[¶]ER is the Ronald S. Hirshberg Chair of Pancreatic Cancer Research, to whom correspondence should be addressed at: 900 Veteran Avenue, Warren Hall Room 11-124, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095-1786, Tel.: (310) 794-6610, erzengurt@mednet.ucla.edu.

INTRODUCTION

Ductal adenocarcinoma of the pancreas is a devastating disease, with overall 5-year survival rate of only 3–5%. The incidence of pancreatic cancer in the US has increased recently to more than 42,000 new cases each year and is now the 4th leading cause of cancer mortality in both men and women (Jemal et al., 2009). As the current therapies offer very limited survival benefits, novel therapeutic strategies are urgently required to treat this aggressive disease. At least one strategy is targeting components of signal transduction pathways that mediate cancer cell proliferation.

Protein kinase D (PKD) is an evolutionarily conserved protein kinase with structural, enzymological, and regulatory properties different from the PKC family members (Johannes et al., 1994; Valverde et al., 1994; Van Lint et al., 1995). The most distinct characteristics of PKD are the presence of a catalytic domain distantly related to Ca²⁺-regulated kinases and a pleckstrin homology (PH) domain that regulates enzyme activity (Iglesias and Rozengurt, 1998; Rozengurt et al., 1995; Rozengurt et al., 1997; Waldron et al., 1999). The subsequent identification of PKD2 (Sturany et al., 2001) and PKD3 (Hayashi et al., 1999; Rey et al., 2003), similar in overall structure and amino acid sequence to PKD (henceforth called PKD1), confirmed the notion that PKD1 is the founding member of a new family of serine/threonine protein kinases (Rozengurt et al., 2005).

PKD1 can be activated within intact cells by multiple stimuli acting through receptor-mediated pathways, including G protein-coupled receptor (GPCR) agonists that act through G_q, G₁₂, G_i, and Rho [reviewed in (Rozengurt et al., 2005)]. We identified Ser⁷⁴⁴ and Ser⁷⁴⁸ in the PKD1 activation loop as phosphorylation sites critical for PKD1 activation (Iglesias et al., 1998; Rozengurt et al., 2005; Waldron et al., 2001; Waldron and Rozengurt, 2003; Yuan et al., 2000). Recent studies demonstrated that rapid PKC-dependent PKD1 activation is followed by a PKC-independent phase of catalytic activation and phosphorylation induced by stimulation of G_q-coupled receptors (Jacamo et al., 2008). Accumulating evidence suggest that PKD family members play a role in the regulation of several cellular processes and activities, including chromatin organization, Golgi function, gene expression, cell survival, adhesion, motility, differentiation, DNA synthesis and proliferation, [reviewed in Ref. (Rozengurt et al., 2005)]. The pleiotropic function of PKD1 signaling appears to be mediated, at least in part, by modulating the intensity and duration of the major MAPK signaling modules (Brandlin et al., 2002; Sinnett-Smith et al., 2004; Sinnett-Smith et al., 2007; Wang et al., 2002), including the extra-cellular signal regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) pathways. Despite considerable progress in elucidating mechanisms of PKD family regulation and function, the precise role of PKD1 signaling in human cancer cells remains poorly characterized.

GPCRs and their cognate agonists are increasingly implicated as autocrine/paracrine growth factors for multiple solid tumors, including pancreatic ductal adenocarcinoma (Dorsam and Gutkind, 2007; Rozengurt, 2002; Rozengurt, 2007). A variety of GPCR agonists, including neurotensin (NT), stimulate DNA synthesis in human pancreatic adenocarcinoma cell lines (Guha et al., 2003; Guha et al., 2002; Kisfalvi et al., 2009; Kisfalvi et al., 2005; Kisfalvi et al., 2007; Ryder et al., 2001). Other studies demonstrated that the NT receptor is commonly expressed in pancreatic cancer specimens (Ehlers et al., 2000; Reubi et al., 1998; Wang et al., 2000) and was identified as a hit from the Cancer Genome Anatomy Project database (Elek et al., 2000). NT binding to its cognate GPCR induces PKD activation, stimulates ERK and p38 MAP kinase signaling and subsequently promotes DNA synthesis and cell proliferation in PANC-1 cells (Guha et al., 2003; Kisfalvi et al., 2009; Kisfalvi et al., 2005; Ryder et al., 2001). In fibroblasts, we demonstrated that overexpression of PKD1 potentiates DNA synthesis induced by GPCR agonists via sustained MEK/ERK/p90RSK signaling

(Sinnott-Smith et al., 2009; Sinnott-Smith et al., 2004; Sinnott-Smith et al., 2007; Zhukova et al., 2001) and PKD1 was reported to be overexpressed in pancreatic cancer tissues (Trauzold et al., 2003). Although PKD1 enhances ERK activation, we and others have demonstrated suppression of JNK/c-Jun signaling by PKD1 in multiple cell types (Bagowski et al., 1999; Hurd and Rozengurt, 2001; Hurd et al., 2002), raising the attractive possibility that PKD1 regulates the balance of pro-mitogenic ERK and pro-apoptotic JNK/c-Jun signaling, at least in some cell types. However, the role of PKD1 in regulating NT-mediated MAP kinase pathways, DNA synthesis and proliferation in human pancreatic adenocarcinoma cells harboring an activating Ras mutation remains poorly defined.

Here, we used inducible stable expression of wild-type (WT) and kinase-dead (K618N) forms of PKD1 in PANC-1 cells to elucidate its function in the regulation of NT-induced MAP kinases, DNA synthesis and cell proliferation. A salient feature of our results is that induced expression of WT PKD1 suppressed pro-apoptotic JNK/c-Jun activation and increased the duration of pro-mitogenic ERK in NT-stimulated PANC-1 cells. We also show that induced expression of WT PKD1 (but not kinase-dead PKD1) in PANC-1 cells stimulated DNA synthesis and proliferation (anchorage-dependent and anchorage-independent growth). Furthermore, WT PKD1 strikingly potentiated NT-induced DNA synthesis. Collectively, our results support the notion that PKD1 is a potential target for therapeutic intervention in pancreatic cancer.

MATERIALS and METHODS

Stable inducible PANC-1 clones

Generation of wild-type pINDsp1-PKD1 (WT) and kinase-dead pINDsp1-PKD1-K618N (K618N) plasmids and stable inducible PANC-1 clones expressing WT or K618N forms of PKD1 have been previously described (Hurd and Rozengurt, 2003; Yuan and Rozengurt, 2008).

Cell culture

PANC-1 and stable inducible PANC-1 clones expressing WT or K618N forms of PKD1 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 400 µg/ml zeocin and hygromycin-B supplemented with 10% fetal bovine serum (FBS). For screening PANC-1 clones, ethanol vehicle (0.25% final concentration) or ponasterone-A (ecdysone) dissolved in ethanol (2.5 µM ponasterone-A final concentration) was added and analyzed by Western blotting using polyclonal PKD antibody (C-20).

Western blot analysis

Confluent PANC-1 cells and stable inducible PANC-1 clones expressing WT or K618N forms of PKD1 were lysed in 500 µl of 2 × SDS-PAGE sample buffer. Whole cell lysates from approximately 2×10^5 cells (100 µl) were resolved by SDS-PAGE on 10% gels, and transferred to PVDF membranes at 400 mA for 4.5 h. The membranes were blocked for 1 h in PBS containing 0.1% Tween-20 (PBS-T) and 5 % nonfat dried milk. Western blotting was then performed with 1:1000 dilution of primary antibody for 2 h in PBS-T containing 3% nonfat dried milk. The immunoreactive bands were next detected with enhanced chemiluminescence reagents (Amersham, UK).

[³H]-Thymidine incorporation into DNA

Confluent PANC-1 cells and stable inducible PANC-1 expressing WT or K618N forms of PKD1 (5×10^4 cells) were plated and grown in 3.5 cm tissue culture plates for 5 days in DMEM with 2 mM glutamine, 1 mM Na-pyruvate and 10% FBS. The cells were washed twice with phosphate-buffered saline (PBS) and incubated in DMEM for 24 h. To start the

experiment, the cultures (4–6 cultures used for each condition) were washed twice with PBS and transferred to fresh medium containing the specified concentration of the inducer ponasterone-A. After 24 h of incubation, the cultures were pulse-labeled for 6 h with [³H]-thymidine (0.25 μ Ci/ml) or treated with 5 nM NT for 17 h and then pulse-labeled for 6 h with [³H]-thymidine, as specified in the individual experiments. Then, the cells were fixed with 5% trichloroacetic acid and washed twice with ethanol. Acid-insoluble pools were dissolved in 0.1 N NaOH containing 1% SDS and counted in a liquid scintillation counter.

Anchorage-Dependent Cell Proliferation

Cultures of PANC-1 cells and stable inducible PANC-1 expressing WT or K618N forms of PKD1, 3–5 days after passage, were washed and suspended in DMEM. Cells were then disaggregated by two passes through a 19-gauge needle into an essentially single-cell suspension as judged by microscopy. Cell number was determined using a Coulter Counter, and 2×10^4 cells were seeded in DMEM containing 1% FBS. After 24 h incubation at 37°C, ponasterone-A (at concentrations specified in each individual experiment) was added to the medium and the cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for 7–9 days. The total cell count was determined from a minimum of four wells per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension ten times through a 19- and subsequently a 21-gauge needle.

Anchorage-Independent Cell Proliferation

Cells were plated on tissue culture 12-well plates coated with polyhydroxyethylmethacrylate [Poly-(HEMA)]. Under these conditions, cells are denied attachment to the substratum. [Poly-(HEMA)] was dissolved at 5 mg/ml in 95% ethanol and kept at 37°C. Six hundred and twenty five microliters of the dissolved solution was added into the wells and the plates were allowed to dry for at least 2 days at 37°C with lids in place. Before use, [Poly-(HEMA)]-coated plates were washed twice in PBS. Cultures of PANC-1 cells and stable inducible PANC-1 expressing WT or K618N forms of PKD1, 3–5 days after passage, were washed and suspended in DMEM. Cells were then disaggregated by two passes through a 19-gauge needle into an essentially single-cell suspension as judged by microscopy. Cell number was determined using a Coulter Counter, and 2×10^4 cells were seeded in DMEM containing 1% FBS on the [Poly-(HEMA)]-coated dishes. After 24 h incubation at 37°C, ponasterone-A was added as indicated in the corresponding experiments. The cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for 7–9 days and the total cell count was determined from a minimum of four wells per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension ten times through a 19- and subsequently a 21-gauge needle.

Materials

The ecdysone-inducible expression kit, pINDsp1 plasmid, ponasterone-A (ecdysone), hygromycin-B and zeocin were purchased from Invitrogen (Carlsbad, CA). PANC-1 was obtained from American Type Culture Collection (ATCC). Phospho-SEK1/MKK4 Thr²⁶¹, phospho-c-Jun Ser⁶³, c-Jun, phospho-PKD Ser⁷⁴⁴/Ser⁷⁴⁸, an antibody that primarily detects the phosphorylated state of Ser⁷⁴⁴ (Jacamo et al., 2008; Waldron et al., 2001), and phospho-ERK-1/2 Thr²⁰²/Tyr²⁰⁴ (E10) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-PKD (C-20) antibody was purchased from Santa Cruz Biotechnology (Palo Alto, CA). Neurotensin (NT), EGF, DMEM (without sodium pyruvate) and PBS were purchased from Sigma (St. Louis, MO). All other reagents were of the purest grade available.

RESULTS

PKD1 suppresses NT-mediated c-Jun Ser⁶³ phosphorylation in PANC-1 clones

The c-Jun N-terminal kinases (JNK1/2) phosphorylate the N-terminal transactivation domain of c-Jun at Ser⁶³/Ser⁷³, thereby potentiating its ability to induce gene expression (Davis, 2000; Weston and Davis, 2002). As a measure of JNK activation, we first determined NT-induced c-Jun Ser⁶³ phosphorylation in human pancreatic ductal adenocarcinoma cell line PANC-1. Wild type PANC-1 cells were treated with NT and lysed after various times of incubation. The extracts were analyzed by Western blotting using an antibody that selectively detects c-Jun Ser⁶³ phosphorylation. As shown in Fig. 1A, minimal c-Jun phosphorylation was detected in unstimulated PANC-1 cells. NT induced a striking increase in c-Jun Ser⁶³ phosphorylation, in a time-dependent manner in PANC-1 cells. Maximal effect was achieved after 15 min of exposure to NT.

It is known that PKD1 inhibits EGF receptor tyrosine kinase (EGFR)-mediated JNK activation in multiple cell types (Bagowski et al., 1999; Hurd and Rozengurt, 2001). In contrast, nothing is known about the effects of PKD1 on GPCR-mediated JNK1/2 activation in cancer cells. Given the fact that NT induces PKC/PKD activation (Guha et al., 2002; Yuan and Rozengurt, 2008) and JNK/c-Jun activation in PANC-1 cells (Fig. 1A), we determined the effects of PKD1 overexpression on NT-induced JNK/c-Jun signaling pathway in these cells. We used stably transfected PANC-1 cells in which wild-type (WT) or kinase-dead (K618N) forms of PKD1 can be inducibly expressed under the control of a modified ecdysone receptor enhancer/promoter (Hurd and Rozengurt, 2001; Hurd and Rozengurt, 2003; Hurd et al., 2002; Yuan and Rozengurt, 2008). As shown in Fig. 1B, addition of increasing concentrations of the ecdysone receptor agonist ponasterone A (Pon-A) for 24 h produced a striking dose-dependent increase in the expression of PKD1, as shown by Western blotting using an antibody that detects the C-terminal region of this enzyme (C-20, Fig. 1B). Maximal PKD1 expression was elicited by Pon-A at a concentration of 1 μ M.

Initially, we examined the effects of induced expression of WT or K618N PKD1 on NT-induced c-Jun Ser⁶³ phosphorylation. PANC-1 clones with WT (Fig. 1C) or K618N PKD1 (Fig. 1D) were treated without (–) or with (+) 1 μ M Pon-A for 22 h to induce expression of either form of PKD1 and were subsequently challenged with NT for 20 min. Whole cell lysates were prepared and analyzed by western blotting with antibodies against phospho-c-Jun Ser⁶³, total c-Jun and phospho-PKD Ser⁷⁴⁴/Ser⁷⁴⁸. NT potently stimulated activation loop phosphorylation at Ser⁷⁴⁴ of both WT (Fig. 1C) and kinase-dead forms of PKD1 (Fig. 1D), indicating that NT induced transphosphorylation in the activation loop of PKD1 in PANC-1 clones.

Similar to wild type PANC-1 cells, NT also stimulated c-Jun Ser⁶³ phosphorylation in both the PANC-1 clones as demonstrated by increased reactivity to phospho-c-Jun Ser⁶³ antibody and the characteristic upshift of c-Jun (Fig. 1C–D). Interestingly, induced expression of WT (Fig. 1C), but not kinase-dead (K618N) PKD1 (Fig. 1D), suppressed both the c-Jun Ser⁶³ phosphorylation and the upshift of c-Jun protein. In contrast, K618N PKD1 failed to suppress c-Jun Ser⁶³ phosphorylation in PANC-1 clones. These results indicate that induced expression of WT PKD1 suppresses GPCR agonist-induced c-Jun Ser⁶³ phosphorylation in PANC-1 cells.

Suppression of NT-induced c-Jun Ser⁶³ phosphorylation by PKD1 occurs upstream of JNK and correlates with PKD1 activation loop phosphorylation in PANC-1 clones

Similar to other MAPK signaling modules, the JNK pathway is composed of three kinases, which sequentially phosphorylate and activate a downstream component. JNK is activated

via dual phosphorylation by MKK4 and MKK7 (Davis, 2000). MKK4, a dual-specificity kinase that can function as a tumor suppressor (Whitmarsh and Davis), is activated by phosphorylation at Thr²⁶¹ (and Ser²⁵⁷) by a variety of upstream kinases, including MEKK1 (Whitmarsh and Davis). Having established that PKD1 suppresses NT-induced c-Jun Ser⁶³ phosphorylation in PANC-1 clones, we examined whether PKD1 can attenuate phosphorylation of MKK4 at Thr²⁶¹. PANC-1 clones with WT PKD were treated without (–) or with (+) 1 μ M Pon-A for 24 h and then challenged with NT for the indicated time-periods (Fig. 2A). The cell lysates were analyzed by Western blotting with antibodies to phospho-MKK4 Thr²⁶¹ and phospho-c-Jun Ser⁶³ (Fig. 2A). Stimulation of PANC-1 cells with NT rapidly induced phosphorylation of MKK4 Thr²⁶¹ and c-Jun Ser⁶³ in a time-dependent manner. Maximal effect was achieved after 20 min of exposure to NT. However, induction of WT PKD1 strikingly attenuated phosphorylation of MKK4 Thr²⁶¹ and c-Jun Ser⁶³ after both 5 and 20 min stimulation with NT in parallel cultures (Fig. 2A). Thus, our results suggest that PKD1 mediates sustained suppression of NT-induced JNK/c-Jun activation in PANC-1 clones either at the level or upstream of MKK4.

Next we determined whether attenuation of NT-induced c-Jun phosphorylation by PKD1 correlates with its activation loop phosphorylation in PANC-1 clones. As shown in Fig. 2A, the previous cell lysates from PANC-1 clones were now probed with antibodies to phospho-PKD Ser⁷⁴⁴ and PKD (C-20). NT-induced maximal PKD activation loop phosphorylation was achieved within 5 min and persisted for up to 20 min. Western blotting with PKD (C-20) antibody confirmed equal amount of total PKD1 in the induced PANC-1 clones. Thus far, our results demonstrate that PKD1 suppresses GPCR-mediated JNK signaling in PANC-1.

NT-induced c-Jun Ser⁶³ phosphorylation is independent of EGFR tyrosine kinase activity in PANC-1 clones

EGFR tyrosine autophosphorylation by GPCR-induced transactivation can lead to activation of MAP kinase modules, at least in some cell types (Rozengurt, 2007). As mentioned previously, PKD1 blocked EGF-induced JNK activation in multiple cell types (Bagowski et al., 1999; Hurd and Rozengurt, 2001). It was therefore conceivable that PKD1 suppressed JNK/c-Jun via inhibition of GPCR-mediated EGFR transactivation. However, NT failed to stimulate any detectable EGFR tyrosine phosphorylation in PANC-1 cells (Santiskulvong and Rozengurt, 2007) and accordingly, NT-induced ERK activation was not prevented by inhibition of EGFR tyrosine kinase activity in these cells (Guha et al., 2003). Consequently, we hypothesized that NT-induced JNK/c-Jun does not depend on EGFR kinase activity in PANC-1 cells.

To test this possibility, PANC-1 clones were treated with Pon-A for 24 h to induce WT PKD expression (Fig. 2B). Next, the clones were treated with the EGFR tyrosine kinase inhibitor AG1478 for 1 h and subsequently challenged with NT for 20 min. As shown in Fig. 2B, NT potently stimulated c-Jun Ser⁶³, ERK-1/2 Thr²⁰²/Tyr²⁰⁴ and PKD1 Ser⁷⁴⁴ phosphorylation in PANC-1 clones as detected by Western blot. Pretreatment with AG1478, at a concentration (250 nM) that completely blocked EGF-induced ERK-1/2 Thr²⁰²/Tyr²⁰⁴ phosphorylation in parallel cultures (results not shown), did not prevent NT-induced ERK-1/2 Thr²⁰²/Tyr²⁰⁴ or c-Jun Ser⁶³ phosphorylation in PANC-1 clones. Our results indicate that the EGFR tyrosine kinase activity is not required for NT-induced ERK-1/2 Thr²⁰²/Tyr²⁰⁴ or c-Jun Ser⁶³ phosphorylation in PANC-1 clones.

Interestingly, inducible expression of WT PKD1 resulted in a detectable increase in NT-induced ERK-1/2 Thr²⁰²/Tyr²⁰⁴ phosphorylation with concomitant suppression of c-Jun Ser⁶³ phosphorylation (Fig. 2B). Also, NT-induced activation loop phosphorylation of PKD1 was enhanced in induced PANC-1 clones and correlated with ERK activation and c-

Jun suppression. Western blotting with PKD (C-20) antibody confirmed equal amount of total PKD1 in the induced PANC-1 clones.

In order to substantiate that PKD1 enhances NT-induced ERK activation, we determined the effect of induced PKD1 expression on ERK activation in PANC-1 cells stimulated with NT for various times. As shown in Fig 2C, inducible expression of WT PKD1 resulted in a striking increase in the duration of NT-induced ERK activation. Collectively, our results indicate that inducible expression of WT PKD1 in PANC-1 clones has a dual effect on NT signaling: significant prolongation of ERK1/2 activation with concomitant suppression of JNK-mediated phosphorylation of c-Jun on Ser⁶³.

PKD1 stimulates DNA synthesis and cell proliferation in PANC-1 cells

The reciprocal influence of PKD1 signaling on ERK and JNK pathways prompted us to examine whether induced expression of PKD1 can promote DNA synthesis and proliferation in PANC-1 cells. In order to determine whether expression of PKD1 stimulates DNA synthesis in PANC-1 cells, cultures of these cells grown in medium containing 10% FBS were transferred to serum-free medium for 24 h. To start the experiment, PANC-1 with WT or K618N PKD1 were treated without or with 1 μ M Pon-A to induce expression of either form of PKD1. After 24 h of incubation, the cultures were pulse-labeled for 6 h with [³H]-thymidine and the incorporation of radioactivity into DNA was measured. As shown in Fig. 3, expression of WT PKD1 in PANC-1 cells induced by 1 μ M Pon-A stimulated a significant increase ($p < 0.005$) in the incorporation of [³H]-thymidine into DNA. In contrast, a similar treatment with Pon-A did not induce any significant increase in DNA synthesis in cells expressing kinase-dead PKD1.

To further substantiate that PKD1 overexpression stimulates proliferation in PANC-1, we also measured cell numbers in response to addition of Pon-A in PANC-1 cells. As shown in Fig. 3B, induction of WT PKD1 (but not K618N PKD1) resulted in a marked increase in the number of PANC-1 cells.

Subsequently, we determined whether PKD1 overexpression leads to the proliferation of PANC-1 cells in the absence of adhesion signals, a hallmark of malignant transformation. Cultures of PANC-1 cells with WT or K618N PKD1 were plated on tissue culture plates coated with polyhydroxyethylmethacrylate [Poly-(HEMA)], prepared as previously described (Kisfalvi et al., 2005). Cells were seeded in DMEM containing 1% FBS on the [Poly-(HEMA)]-coated dishes. After incubation for 24 h, the cells were treated without or with 1 μ M Pon-A to induce PKD1 expression. The cultures were incubated for 7 days and the cell count for each condition was determined from at least 4 wells. The results, shown in Fig. 3C, demonstrate that expression in PANC-1 cells of WT but not kinase-dead mutant PKD1, stimulated a significant increase in the number of these cells cultured in [Poly-(HEMA)]-coated dishes.

We verified that treatment of stable inducible PANC-1 expressing WT PKD1 with increasing concentrations of Pon-A to induce increasing levels of PKD1 expression (as shown in Fig. 1B) stimulated DNA synthesis (Fig. 4A) and cell proliferation (Fig. 4B) in a concentration-dependent manner. The maximal effect was achieved at a Pon-A concentration of 1 μ M. Collectively these results illustrate, for the first time, that induced expression of WT PKD1 leads to DNA synthesis and proliferation in a model of human pancreatic carcinoma cells.

PKD1 mediates NT-induced DNA synthesis in PANC-1 cells

Next, we determined whether induced expression of PKD1 potentiates NT-stimulated DNA synthesis in pancreatic cancer cells. Confluent cultures of PANC-1 cells and stable inducible

PANC-1 expressing WT PKD1 or K618N PKD1 were treated with 1 μ M Pon-A. After 24 hr incubation, the cultures were washed and transferred to DMEM with 1 μ M Pon-A and different concentrations of NT. As shown in Fig. 5, treatment of PANC-1 cells with 5 nM NT stimulated a moderate increase in DNA synthesis (from 3.9 $\pm$ 0.2 to 7.1 $\pm$ 0.32). In the PANC-1 expressing WT PKD1 and treated with 1 μ M Pon-A, the stimulation of DNA synthesis induced by 5 nM NT was strikingly increased (to 15.8 $\pm$ 0.22). The level of DNA synthesis obtained was higher than that expected for an additive response (marked by the arrows), indicating that NT and PKD1 act synergistically to induce DNA synthesis in PANC-1 cells. In contrast, addition of Pon-A to PANC-1 cells expressing K618N PKD1 did not produce any significant enhancement of NT-induced DNA synthesis (Fig. 5).

DISCUSSION

PKD1 has been reported recently to mediate several important cellular activities and processes, including cell survival, migration, differentiation, DNA synthesis and proliferation [reviewed in Ref. (Rozengurt et al., 2005)]. Thus, mounting evidence indicates that PKD1 has a remarkable diversity of both its signal generation and distribution and its potential for complex regulatory interactions with multiple downstream pathways leading to multiple responses, including long-term cellular events. However, PKD1 mediated downstream effects on MAPK signaling pathways and proliferation in cancer cells harboring an activating Ras mutation has remained poorly characterized.

NT binding to its cognate receptor (NTR-1) induces Gq-mediated potent activation of protein kinase C (PKC)/protein kinase D (PKD) signaling pathway in human pancreatic carcinoma PANC-1 cells (Guha et al., 2002; Yuan and Rozengurt, 2008). Our previous studies indicated that PKC-mediated ERK activation enhances cell cycle progression and promotes colony formation on soft agar in these cells (Guha et al., 2003; Guha et al., 2002). The results presented here demonstrate that NT also induces JNK/c-Jun activation in PANC-1 cells. We found that induced expression of WT PKD1 suppressed NT-induced c-Jun Ser⁶³ phosphorylation and the upshift of c-Jun protein. In contrast, K618N PKD1 (kinase-dead) failed to suppress c-Jun Ser⁶³ phosphorylation in PANC-1 clones. This strongly indicates that the catalytic activity of PKD1 is required for suppression of GPCR agonist-induced c-Jun Ser⁶³ phosphorylation in pancreatic cancer cells. Induced expression of PKD1 markedly attenuated NT mediated JNK/c-Jun activation at or upstream of MKK4, a putative tumor suppressor (Whitmarsh and Davis), though effects of PKD1 at additional levels cannot be eliminated (Hurd et al., 2002; Waldron et al., 2007). Although PKD1 was implicated in attenuation of EGF-induced JNK signaling (Bagowski et al., 1999; Hurd and Rozengurt, 2001; Hurd et al., 2002), to our knowledge, this is the first demonstration that PKD1 suppresses GPCR agonist mediated MKK4/JNK/c-Jun signaling pathway in pancreatic cancer cells.

In contrast to the attenuating effect of PKD1 on JNK/c-Jun activation, our studies show that PKD1 increased the duration of ERK signaling in human pancreatic carcinoma PANC-1 cells. These results reinforce the notion that an activating Ras mutation, as present in PANC-1 cells, is not sufficient to constitutively activate the ERK pathway.

It is increasingly recognized that the duration and intensity of activation of the MAP kinase pathways are of critical importance for determining specific biological outcomes, including cell proliferation and the induction of cell death (Marshall, 1995; Pouyssegur and Lenormand, 2003; Rozengurt, 2007; Wagner and Nebreda, 2009). Although biological outcomes depend on stimulus and cell type, transient JNK activation was shown to promote cell survival while prolonged JNK activation mediates apoptosis (Wagner and Nebreda, 2009). Consequently, it is plausible that the attenuation of JNK/c-Jun activation mediated by

PKD1 facilitates survival and proliferation of pancreatic cancer cells. Conversely, sustained (rather than transient) ERK signaling has been linked to stimulation of cell proliferation in several cell types (Murphy et al., 2004; Pouyssegur and Lenormand, 2003; Sinnett-Smith et al., 2004; Sinnett-Smith et al., 2007), including pancreatic cancer cells (Kisfalvi et al., 2005). Taken together, these studies underscore one of the major emerging roles of PKD1, namely concomitant upregulation of GPCR-mediated mitogenic ERK signaling and downregulation of sustained pro-apoptotic JNK signaling pathway.

In view of the reciprocal influence of PKD1 on MKK4/JNK/c-Jun and ERK pathways in human pancreatic carcinoma cells, we hypothesized that induced PKD1 expression promotes pancreatic carcinoma cell proliferation. Here, we produced several lines of evidence indicating that PKD1 induces mitogenic signaling in human pancreatic carcinoma PANC-1 cells: 1) expression of WT PKD1 in PANC-1 cells induced by Pon-A stimulated a significant increase in the incorporation of [³H]-thymidine into DNA. In contrast, a similar treatment with the inducer did not induce any significant increase in DNA synthesis in cells expressing kinase-dead PKD1; 2) expression in PANC-1 cells of WT but not kinase-dead mutant PKD1, stimulated a significant increase in the number of these cells cultured in either regular plastic dishes or in [Poly-(HEMA)]-coated dishes (i.e. anchorage-dependent and anchorage-independent conditions, respectively). These results suggest that PKD1 promotes proliferation in the absence of adhesion-mediated signals, a hallmark of malignantly transformed cells; 3) PKD1-induced DNA synthesis and cell proliferation were proportional to the levels of its expression, as produced by adding increasing concentrations of the inducer Pon-A; 4) NT stimulation of DNA synthesis was strikingly increased by expression of PKD1. Indeed, the level of DNA synthesis achieved by increasing concentrations of NT in PKD1 expressing PANC-1 cells was higher than that expected for a simple additive response, indicating that NT and PKD1 act synergistically to induce DNA synthesis in PANC-1 cells. The synergistic effect is likely to reflect NT-stimulated PKD1 activation and a rate-limiting role of PKD1 in NT-induced mitogenesis. Collectively, these results indicate that PKD1 is a downstream signaling element that promotes proliferation in PANC-1 cells, an extensively used model of human pancreatic carcinoma cells.

In conclusion, our results indicate that PKD1 overexpression leads to reciprocal regulation of NT-induced MAPK pathways in PANC-1 cells. Specifically, PKD1 suppresses MKK4/JNK/c-Jun Ser⁶³ phosphorylation and concomitantly prolongs ERK1/2 activation. A salient feature of the results shown here is that treatment of stable inducible PANC-1 expressing WT PKD1 with Pon-A to induce increasing levels of PKD1 expression stimulated DNA synthesis, anchorage-dependent and anchorage-independent PANC-1 proliferation and markedly enhanced NT-induced DNA synthesis in these cells. Thus, PKD1 emerges as a potential novel target for developing therapeutic strategies to restrict the unregulated proliferation of pancreatic cancer cells.

Abbreviations used

EGF	epidermal growth factor
PKC	protein kinase C
PKD1	protein kinase D1
NT	neurotensin
JNK	c-Jun N-terminal kinase
ERK	extra-cellular signal-regulated kinase
MAPK	mitogen-activated protein kinase

MEK	mitogen-activated and extracellular signal-regulated kinase kinase
MEKK1	MEK kinase 1
MKK4	mitogen-activated protein kinase kinase 4
GPCR	G protein-coupled receptor
Pon-A	Ponasterone A
PBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum

References

- Bagowski CP, Stein-Gerlach M, Choidas A, Ullrich A. Cell-type specific phosphorylation of threonines T654 and T669 by PKD defines the signal capacity of the EGF receptor. *EMBO J.* 1999; 18:5567–5576. [PubMed: 10523301]
- Brandlin I, Hubner S, Eiseler T, Martinez-Moya M, Horschinek A, Hausser A, Link G, Rupp S, Storz P, Pfizenmaier K, Johannes FJ. Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways. *J Biol Chem.* 2002; 277:6490–6496. [PubMed: 11741879]
- Davis RJ. Signal Transduction by the JNK Group of MAP Kinases. *Cell.* 2000; 103:239–252. [PubMed: 11057897]
- Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer.* 2007; 7:79–94. [PubMed: 17251915]
- Ehlers RA, Kim SH, Zhang YJ, Ethridge RT, Murrilo C, Hellmich MR, Evans DB, Townsend CM, Evers BM. Gut peptide receptor expression in human pancreatic cancers. *Ann Surg.* 2000; 231:838–846. [PubMed: 10816627]
- Elek J, Pinzon W, Park KH, Narayanan R. Relevant genomics of neurotensin receptor in cancer. *Anticancer Res.* 2000; 20:53–58. [PubMed: 10769634]
- Guha S, Lunn JA, Santiskulvong C, Rozengurt E. Neurotensin Stimulates Protein Kinase C-dependent Mitogenic Signaling in Human Pancreatic Carcinoma Cell Line PANC-1. *Cancer Res.* 2003; 63:2379–2387. [PubMed: 12750255]
- Guha S, Rey O, Rozengurt E. Neurotensin Induces Protein Kinase C-dependent Protein Kinase D Activation and DNA Synthesis in Human Pancreatic Carcinoma Cell Line PANC-1. *Cancer Res.* 2002; 62:1632–1640. [PubMed: 11912133]
- Hayashi A, Seki N, Hattori A, Kozuma S, Saito T. PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. *Biochim Biophys Acta.* 1999; 1450:99–106. [PubMed: 10231560]
- Hurd C, Rozengurt E. Protein kinase D is sufficient to suppress EGF-induced c-Jun Ser 63 phosphorylation. *Biochem Biophys Res Commun.* 2001; 282:404–408. [PubMed: 11401472]
- Hurd C, Rozengurt E. Uncoupling of protein kinase D from suppression of EGF-dependent c-Jun phosphorylation in cancer cells. *Biochem Biophys Res Commun.* 2003; 302:800–804. [PubMed: 12646240]
- Hurd C, Waldron RT, Rozengurt E. Protein kinase D complexes with C-Jun N-terminal kinase via activation loop phosphorylation and phosphorylates the C-Jun N-terminus. *Oncogene.* 2002; 21:2154–2160. [PubMed: 11948398]
- Iglesias T, Rozengurt E. Protein kinase D activation by mutations within its pleckstrin homology domain. *J Biol Chem.* 1998; 273:410–416. [PubMed: 9417097]
- Iglesias T, Waldron RT, Rozengurt E. Identification of in vivo phosphorylation sites required for protein kinase D activation. *J Biol Chem.* 1998; 273:27662–27667. [PubMed: 9765302]
- Jacamo R, Sinnott-Smith J, Rey O, Waldron RT, Rozengurt E. Sequential Protein Kinase C (PKC)-dependent and PKC-independent Protein Kinase D Catalytic Activation via Gq-coupled Receptors:

DIFFERENTIAL REGULATION OF ACTIVATION LOOP SER744 AND SER748 PHOSPHORYLATION. *J Biol Chem.* 2008; 283:12877–12887. [PubMed: 18337243]

- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer Statistics, 2009. *CA Cancer J Clin.* 2009; 59:225–249. [PubMed: 19474385]
- Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K. PKC α is a novel, atypical member of the protein kinase C family. *J Biol Chem.* 1994; 269:6140–6148. [PubMed: 8119958]
- Kisfalvi K, Eibl G, Sinnott-Smith J, Rozengurt E. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. *Cancer Res.* 2009; 69:6539–6545. [PubMed: 19679549]
- Kisfalvi K, Guha S, Rozengurt E. Neurotensin and EGF induce synergistic stimulation of DNA synthesis by increasing the duration of ERK signaling in ductal pancreatic cancer cells. *J Cell Physiol.* 2005; 202:880–890. [PubMed: 15389644]
- Kisfalvi K, Rey O, Young SH, Sinnott-Smith J, Rozengurt E. Insulin Potentiates Ca²⁺ Signaling and Phosphatidylinositol 4,5-Bisphosphate Hydrolysis Induced by Gq Protein-Coupled Receptor Agonists through an mTOR-Dependent Pathway. *Endocrinology.* 2007; 148:3246–3257. [PubMed: 17379645]
- Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 1995; 80:179–185. [PubMed: 7834738]
- Murphy LO, MacKeigan JP, Blenis J. A Network of Immediate Early Gene Products Propagates Subtle Differences in Mitogen-Activated Protein Kinase Signal Amplitude and Duration. *Mol Cell Biol.* 2004; 24:144–153. [PubMed: 14673150]
- Pouyssegur J, Lenormand P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Eur J Biochem.* 2003; 270:3291–3299. [PubMed: 12899687]
- Reubi JC, Waser B, Friess H, Beuchler M, Laissue J. Neurotensin receptors: a new marker for human ductal pancreatic adenocarcinoma. *Gut.* 1998; 42:546–550. [PubMed: 9616318]
- Rey O, Yuan J, Young SH, Rozengurt E. Protein kinase C α /protein kinase D3 nuclear localization, catalytic activation, and intracellular redistribution in response to G protein-coupled receptor agonists. *J Biol Chem.* 2003; 278:23773–23785. [PubMed: 12676944]
- Rozengurt E. Neuropeptides as growth factors for normal and cancer cells. *Trends Endocrinol Metabol.* 2002; 13:128–134.
- Rozengurt E. Mitogenic signaling pathways induced by G protein-coupled receptors. *J Cell Physiol.* 2007; 213:589–602. [PubMed: 17786953]
- Rozengurt E, Rey O, Waldron RT. Protein Kinase D Signaling. *J Biol Chem.* 2005; 280:13205–13208. [PubMed: 15701647]
- Rozengurt E, Sinnott-Smith J, Van Lint J, Valverde AM. Protein kinase D (PKD): a novel target for diacylglycerol and phorbol esters. *Mutat Res.* 1995; 333:153–160. [PubMed: 8538623]
- Rozengurt E, Sinnott-Smith J, Zugaza JL. Protein kinase D: a novel target for diacylglycerol and phorbol esters. *BochemSoc Trans.* 1997; 25:565–571.
- Ryder NM, Guha S, Hines OJ, Reber HA, Rozengurt E. G protein-coupled receptor signaling in human ductal pancreatic cancer cells: Neurotensin responsiveness and mitogenic stimulation. *J Cell Physiol.* 2001; 186:53–64. [PubMed: 11147814]
- Santiskulvong C, Rozengurt E. Protein kinase C α mediates feedback inhibition of EGF receptor transactivation induced by G(q)-coupled receptor agonists. *Cell Signal.* 2007; 19:1348–1357. [PubMed: 17307332]
- Sinnott-Smith J, Jacamo R, Kui R, Wang YM, Young SH, Rey O, Waldron RT, Rozengurt E. Protein Kinase D Mediates Mitogenic Signaling by Gq-coupled Receptors through Protein Kinase C-independent Regulation of Activation Loop Ser744 and Ser748 Phosphorylation. *J Biol Chem.* 2009; 284:13434–13445. [PubMed: 19289471]
- Sinnott-Smith J, Zhukova E, Hsieh N, Jiang X, Rozengurt E. Protein kinase D potentiates DNA synthesis induced by Gq-coupled receptors by increasing the duration of ERK signaling in swiss 3T3 cells. *J Biol Chem.* 2004; 279:16883–16893. [PubMed: 14963034]
- Sinnott-Smith J, Zhukova E, Rey O, Rozengurt E. Protein kinase D2 potentiates MEK/ERK/RSK signaling, c-Fos accumulation and DNA synthesis induced by bombesin in Swiss 3T3 cells. *J Cell Physiol.* 2007; 211:781–790. [PubMed: 17226786]

- Sturany S, Van Lint J, Mueller F, Wilda M, Hameister H, Hoecker M, Brey A, Gern U, Vandenheede J, Gress T, Adler G, Seufferlein T. Molecular cloning and characterization of the human protein kinase D2: A novel member of the protein kinase D family of serine threonine kinases. *J Biol Chem*. 2001; 276:3310–3318. [PubMed: 11062248]
- Trauzold A, Schmiedel S, Sipos B, Wermann H, Westphal S, Roder C, Klapper W, Arlt A, Lehnert L, Ungefroren H, Johannes FJ, Kalthoff H. PKCmu prevents CD95-mediated apoptosis and enhances proliferation in pancreatic tumour cells. *Oncogene*. 2003; 22:8939–8947. [PubMed: 14654790]
- Valverde AM, Sinnott-Smith J, Van Lint J, Rozengurt E. Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc Natl Acad Sci USA*. 1994; 91:8572–8576. [PubMed: 8078925]
- Van Lint JV, Sinnott-Smith J, Rozengurt E. Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. *J Biol Chem*. 1995; 270:1455–1461. [PubMed: 7836415]
- Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 2009; 9:537. [PubMed: 19629069]
- Waldron RT, Iglesias T, Rozengurt E. The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C. *J Biol Chem*. 1999; 274:9224–9230. [PubMed: 10092595]
- Waldron RT, Rey O, Iglesias T, Tugal T, Cantrell D, Rozengurt E. Activation Loop Ser744 and Ser748 in Protein Kinase D Are Transphosphorylated in Vivo. *J Biol Chem*. 2001; 276:32606–32615. [PubMed: 11410586]
- Waldron RT, Rozengurt E. Protein kinase C phosphorylates protein kinase D activation loop Ser744 and Ser748 and releases autoinhibition by the pleckstrin homology domain. *J Biol Chem*. 2003; 278:154–163. [PubMed: 12407104]
- Waldron RT, Whitelegge JP, Faull KF, Rozengurt E. Identification of a novel phosphorylation site in c-jun directly targeted in vitro by protein kinase D. *Biochem Biophys Res Commun*. 2007; 356:361–367. [PubMed: 17359934]
- Wang L, Friess H, Zhu Z, Graber H, Zimmermann A, Korc M, Reubi JC, Beuchler MW. Neurotensin receptor-1 mRNA analysis in normal pancreas and pancreatic disease. *Clin Cancer Res*. 2000; 6:566–571. [PubMed: 10690540]
- Wang Y, Waldron RT, Dhaka A, Patel A, Riley MM, Rozengurt E, Colicelli J. The RAS Effector RIN1 Directly Competes with RAF and Is Regulated by 14-3-3 Proteins. *Mol Cell Biol*. 2002; 22:916–926. [PubMed: 11784866]
- Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev*. 2002; 12:14–21. [PubMed: 11790549]
- Whitmarsh AJ, Davis RJ. Role of mitogen-activated protein kinase kinase 4 in cancer. *Oncogene*. 2003; 22:3172. [PubMed: 12496914]
- Yuan J, Rozengurt E. PKD, PKD2, and p38 MAPK mediate Hsp27 serine-82 phosphorylation induced by neurotensin in pancreatic cancer PANC-1 cells. *J Cell Biochem*. 2008; 103(2):648–662. [PubMed: 17570131]
- Yuan JZ, Slice L, Walsh JH, Rozengurt E. Activation of protein kinase D by signaling through the alpha subunit of the heterotrimeric G protein G(q). *J Biol Chem*. 2000; 275(3):2157–2164. [PubMed: 10636921]
- Zhukova E, Sinnott-Smith J, Rozengurt E. Protein Kinase D Potentiates DNA Synthesis and Cell Proliferation Induced by Bombesin, Vasopressin, or Phorbol Esters in Swiss 3T3 Cells. *J Biol Chem*. 2001; 276(43):40298–40305. [PubMed: 11514571]

Fig. 1. PKD1-mediated attenuation of NT-induced JNK activation in PANC-1 clones

(A) *NT stimulates JNK in PANC-1 cells: time-course.* Confluent PANC-1 cells were washed twice with PBS and incubated in serum-free DMEM for 6 h. Serum-starved cells were stimulated with 40 nM NT for the indicated time-periods at 37°C. The cultures were then washed in cold PBS, lysed in 2 × SDS-PAGE sample buffer, resolved by 10% SDS-PAGE and transferred to PVDF membranes (as indicated in *Materials and Methods*). Samples were analyzed for c-Jun activation by Western blotting using the phospho-c-Jun Ser⁶³ specific polyclonal antibody. (B) *Stable transfected PANC-1 clones express WT PKD1: dose-response curve.* Confluent cultures of stable transfected PANC-1 clones were treated with increasing doses of the inducer ponasterone-A (Pon-A) in DMEM for 24 h. Cell lysates were analyzed by western blot with antibodies that detect the C terminal of PKD1 (C-20). Shown here are representative immunoblots. Similar results were obtained from three independent experiments. (C and D) *WT PKD1, but not kinase-dead PKD1, suppresses NT-mediated c-Jun Ser⁶³ phosphorylation in PANC-1 cells.* Stable inducible PANC-1 clones that express WT (C) or kinase-dead PKD (K618N) (D) were treated with 5 μM ponasterone-A (+) or ethanol vehicle (−) for 24 h. Cells were then treated with 40 nM NT for 20 min and lysed in 2 × SDS-PAGE sample buffer. The lysates were analyzed by Western blotting with antibodies that detect phospho-c-Jun Ser⁶³ (P-c-Jun Ser⁶³), total c-Jun expression (c-Jun) and PKD1 phosphorylated in its activation loop at Ser⁷⁴⁴ and Ser⁷⁴⁸ (P-PKD Ser^{744/748}). Shown here are representative immunoblots. Similar results were obtained from three independent experiments.

Fig. 2. PKD1 suppresses NT-induced c-Jun Ser⁶³ phosphorylation but increases the duration of NT-induced ERK activation

(A) *PKD1 mediated suppression of NT-induced phosphorylation of JNK/c-Jun Ser⁶³ and MKK4 Thr²⁶¹ correlates with its activation loop phosphorylation in PANC-1 clones.* Stable inducible PANC-1 clones that express WT PKD were treated with 5 μ M ponasterone-A (+) or ethanol vehicle (-) for 24 h and subsequently challenged with 40 nM NT for the indicated time-periods and lysed. The lysates were analyzed by western blot with antibodies that detect phospho-MKK4 Thr²⁶¹ (p-MKK4 Thr²⁶¹), phospho-c-Jun Ser⁶³ (P-c-Jun Ser⁶³), PKD phosphorylated in its activation loop at Ser⁷⁴⁴ and Ser⁷⁴⁸ (P-PKD Ser⁷⁴⁴/Ser⁷⁴⁸) or total PKD expression (C-20). Shown here is a representative immunoblot. Similar results were obtained from three independent experiments. (B) *NT-induced c-Jun Ser⁶³ phosphorylation is independent of EGFR tyrosine kinase activity in PANC-1 clones.* Stable inducible PANC-1 clones that express WT PKD were treated with 5 μ M ponasterone-A (+) or ethanol vehicle (-) for 24 h and subsequently treated with or without AG1478 for 30 min, challenged with 40 nM NT for 20 min and then lysed. The lysates were analyzed by western blot with antibodies that detect phospho-c-Jun Ser⁶³ (P-c-Jun Ser⁶³), pERK-1/2 Thr²⁰²/Tyr²⁰⁴ (P-pERK-1/2 Thr²⁰²/Tyr²⁰⁴), PKD phosphorylated in its activation loop at Ser⁷⁴⁴ (P-PKD Ser⁷⁴⁴/Ser⁷⁴⁸) or total PKD expression (C-20). Shown here is a representative immunoblot. Similar results were obtained from three independent experiments. (C) *PKD1 overexpression increases the duration of NT-induced ERK activation.* Confluent inducible

PANC-1 clones that express WT PKD were incubated in serum-free DMEM in the presence (+) or absence (–) of 1 μ M ponasterone-A for 24 h at 37°C. Then, 5nM NT was added to the cultures and the cells were incubated for the time-periods indicated (0–240 min). Cell lysates were analyzed for ERK activation by Western blot using the phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) specific monoclonal antibody.

Fig. 3. PKD1 increases DNA synthesis, anchorage-dependent and independent growth in PANC-1 cells

(A) *WT but not kinase-dead PKD1 increases [³H]-thymidine incorporation.* Confluent cultures of PANC-1 cells and stably inducible PANC-1 clones that express WT PKD1 or kinase-dead PKD1 were washed with PBS and incubated in serum-free DMEM for 24 h at 37°C. The cells were then washed again with PBS and the medium was replaced with DMEM without (–) (open bars) or with (+) (closed bars) 1 μM ponasterone-A and incubated for another 24 h. The cultures were pulse-labeled with [³H]-thymidine (0.25 μCi/ml) for 6 h and the incorporation of radioactivity was determined as described in Materials and Methods. (B) *WT but not kinase-dead PKD1 increases PANC-1 cell proliferation.* PANC-1 cells and PANC-1 cells stably and inducibly overexpressing WT or kinase-dead PKD1 were seeded on normal tissue culture dishes (2 × 10⁴ single cells). After 24 h incubation at 37°C, the medium was changed to fresh DMEM containing 1% FBS in the presence (closed bars) or absence (open bars) of 1 μM ponasterone-A and the cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for 7–9 days. The total cell count was determined from a minimum of four wells per condition using a Coulter counter, as described in Materials and Methods. The results are expressed as percentage of the control.

(C) *WT but not kinase-dead PKD1 increases PANC-1 cell proliferation in [Poly-(HEMA)]-coated dishes.* Cells were plated on tissue culture plates coated with [Poly-(HEMA)], prepared as described in Materials and Methods. PANC-1 cells and stable inducible PANC-1 expressing WT or K618N forms of PKD1, were seeded in DMEM containing 1% FBS on the [Poly-(HEMA)]-coated dishes (2×10^4 cells). After 24 h incubation at 37°C, 1 μ M ponasterone-A was added, as indicated. The cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for 7–9 days and the total cell count was determined from a minimum of four wells per condition using a Coulter counter, as described in Materials and Methods. The results are expressed as percentage of the control.

Fig. 4. PKD1 expression increases DNA synthesis and cell proliferation in a dose-dependent manner

(A) *WT PKD1 expression dose-dependently induces DNA synthesis PANC-1 cells.* Stably inducible PANC-1 clones that express WT PKD1 were washed with PBS and incubated in serum-free DMEM for 24 h at 37°C and then treated with increasing doses of ponasterone-A for 24 h, as indicated. The cultures then were pulse-labeled with [³H]-thymidine (0.25 μCi/ml) for 6 h and the incorporation of radioactivity was determined as described in Materials and Methods. The results represent three independent experiments (each performed by triplicate). Stars denote the significant difference ($p < 0.005$). (B) *WT PKD1 expression dose-dependently induces anchorage-dependent cell proliferation in PANC-1 cells.* 2×10^4 single cells of stably inducible PANC-1 cells that express WT PKD1 were seeded on normal tissue culture dishes. The cells were incubated for 24 h at 37°C, then the medium was changed to fresh DMEM containing 1% FBS and increasing doses ponasterone-A, as indicated. The cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for

7–9 days. The total cell count was determined from a minimum of four wells per condition using a Coulter counter. The results represent three independent experiments. Stars denote the significant difference ($p < 0.005$)

Fig. 5. PKD1 expression enhances NT-induced DNA synthesis in PANC-1 cells

Confluent cultures of intact PANC-1 cells (open bars) and stably inducible PANC-1 clones that express WT PKD1 (black bars) or kinase-dead PKD1 (gray bars) were washed with PBS and incubated in serum-free DMEM for 24 h at 37°C. The cells were then washed again with PBS and the medium was replaced with fresh serum-free DMEM containing 1 μM ponasterone A (Pon-A). After 24 h, NT was added to the medium at different concentrations (as indicated) and the cultures were incubated for another 17 h. Then, the cells were pulse-labeled with [³H]-thymidine (0.25 μCi/ml) for 6 h and the incorporation of radioactivity into acid-insoluble pools was determined as described in Materials and Methods. The results represent three independent experiments (with 3 cultures per condition). Stars denote the significant difference vs control PANC-1 ($p < 0.005$). The arrows indicate expected additive responses.