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Protein kinase Cι is required for Ras transformation and colon carcinogenesis in vivo

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Protein kinase C ι (PKCι) has been implicated in Ras signaling, however, a role for PKCι in oncogenic Ras-mediated transformation has not been established. Here, we show that PKCι is a critical downstream effector of oncogenic Ras in the colonic epithelium. Transgenic mice expressing constitutively active PKCι in the colon are highly susceptible to carcinogen-induced colon carcinogenesis, whereas mice expressing kinase-deficient PKCι (kdPKCι) are resistant to both carcinogen- and oncogenic Ras-mediated carcinogenesis. Expression of kdPKCι in Ras-transformed rat intestinal epithelial cells blocks oncogenic Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. Constitutively active Rac1 (RacV12) restores invasiveness and anchorage-independent growth in Ras-transformed rat intestinal epithelial cells expressing kdPKCι. Our data demonstrate that PKCι is required for oncogenic Ras- and carcinogen-mediated colon carcinogenesis in vivo and define a procarcinogenic signaling axis consisting of Ras, PKCι, and Rac1.

Introduction

PKCι plays a requisite role in Bcr–Abl-mediated resistance to chemotherapy-induced apoptosis (Murray and Fields, 1997; Jamieson et al., 1999), and is critical for epithelial cell polarity (Suzuki et al., 2002) and cell survival (Murray and Fields, 1997; Jamieson et al., 1999). (PKCι refers to the human gene. The corresponding gene in rodents, which is 95% homologous to PKCι at the amino acid level, is termed PKCλ. For clarity, we will refer to both the human and rodent genes and gene products as PKCι.) Although PKCι has also been implicated in Ras-mediated signaling (Uberall et al., 1999; Coghlan et al., 2000; Kampfer et al., 2001), nothing is known about its role in oncogenic Ras-mediated transformation. Activating Ras mutations occur in ∼30% of all human cancers (Adjei, 2001), and in ∼50% of human colon adenomas and carcinomas (Bos, 1989). Here, we investigate the role of PKCι in Ras-mediated oncogenic transformation. Our data demonstrate that Ras-mediated transformation, invasion, and anchorage-independent growth of intestinal epithelial cells requires PKCι activity. Furthermore, we demonstrate that PKCι is critical for Ras- and carcinogen-mediated colon carcinogenesis in vivo.

Results and discussion

As a first step in examining the role of PKCι in colon carcinogenesis, we assessed the expression of PKCι in normal mouse colonic epithelium and azoxymethane (AOM)-induced colon tumors. Immunoblot analysis demonstrated that PKCι is elevated in colon tumors compared with matched, uninvolved epithelium (Fig. 1 a). RT-PCR analysis demonstrated a corresponding increase in PKCι mRNA in colon tumors (Fig. 1 b). PKCι was also elevated in human colon carcinoma specimens when compared with matched uninvolved colonic epithelium (Fig. 1 c), demonstrating that elevated PKCι is a common feature of both mouse and human colon tumors. Immunohistochemical staining confirmed the elevated expression of PKCι in mouse colon tumors (Fig. 2 b) compared

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Abbreviations used in this paper: ACF, aberrant crypt foci; AOM, azoxymethane; caPKCι, constitutively active PKCι; kdPKCι, kinase-deficient PKCι; RacN17, dominant negative Rac1; RacV12, constitutively active Rac1; RIE, rat intestinal epithelial; RIE/Ras, Ras-transformed RIE.

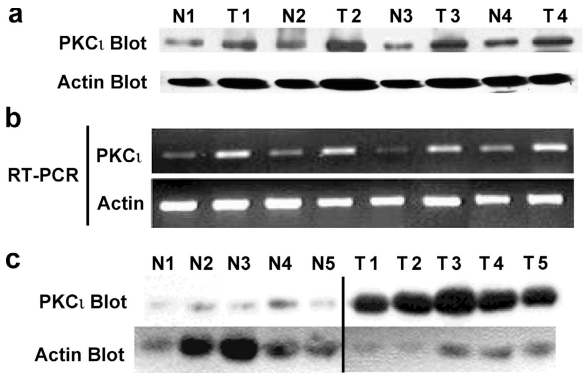

Figure 1. **PKCι is elevated mouse and human colon tumors.** (a) Total protein lysates and (b) total RNA extracts were prepared from AOM-induced mouse colon tumors and uninvolved colonic epithelium from the same animals as described previously (Gökmen-Polar et al., 2001). (a) Immunoblot analysis for PKCι and actin. (b) RT-PCR analysis for mouse PKCι and actin mRNA. Lanes N1–N4 indicate uninvolved mouse colonic epithelium; T1–T4 indicate mouse colon tumors. (c) Immunoblot analysis of lysates from colon tumor tissue and matched, uninvolved colonic epithelium from five patients with colon carcinoma. Equal amounts of protein (50 μg) were subjected to immunoblot analysis for PKCι and actin. Lanes N1–N5 indicate uninvolved human colonic epithelium; lanes T1–T5 indicate matched human colon tumors.

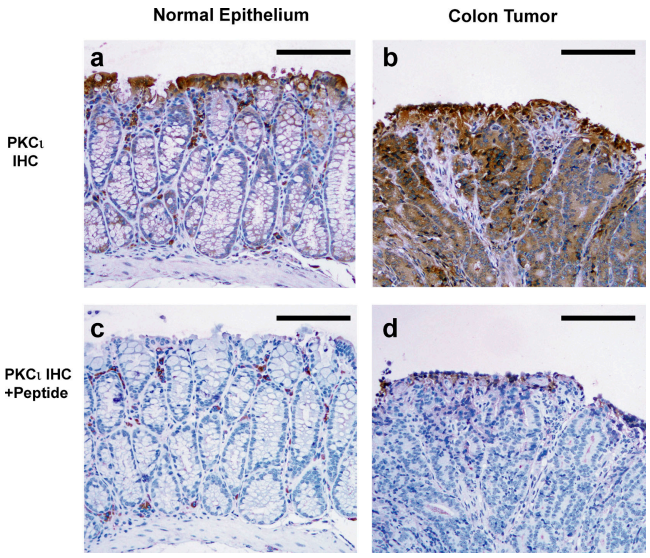

Figure 2. **PKCι expression is elevated in AOM-induced colon tumors.** Immunohistochemical analysis of sections from (a and c) normal, uninvolved epithelium and (b and d) an AOM-induced colon tumor from the same animal was performed using a specific PKCι antibody in the (a and b) absence or (c and d) presence of competing PKCι peptide as described in Materials and methods. Bars, 50 μm.

with normal colonic epithelium (Fig. 2 a). Specificity of the immunostaining was confirmed by staining with antibody in the presence of a fivefold molar excess of the PKCι peptide used to generate the PKCι antibody (Fig. 2, c and d).

The elevated expression of PKCι in colon tumors suggested that PKCι may play an important role in colon carcinogenesis. To test this hypothesis, we generated transgenic mice that express either a constitutively active PKCι (caPKCι) or kinase-deficient PKCι (kdPKCι) in the colonic epithelium. Transgenic caPKCι and kdPKCι mice express elevated PKCι protein in the colonic epithelium (Fig. 3, a and b, top). Transgenic caPKCι mice exhibited high intrinsic PKCι activity in the colonic epithelium when compared with nontransgenic littermates (Fig. 3 a, bottom). In contrast, transgenic kdPKCι mice exhibited decreased colonic PKCι kinase activity when compared with nontransgenic littermates (Fig. 3 b, bottom; the autoradiograph in Fig. 3 b is a longer exposure than Fig. 3 a to reveal the decreased PKCι activity in transgenic kdPKCι mice). Neither transgenic caPKCι nor transgenic kdPKCι mice exhibited demonstra-

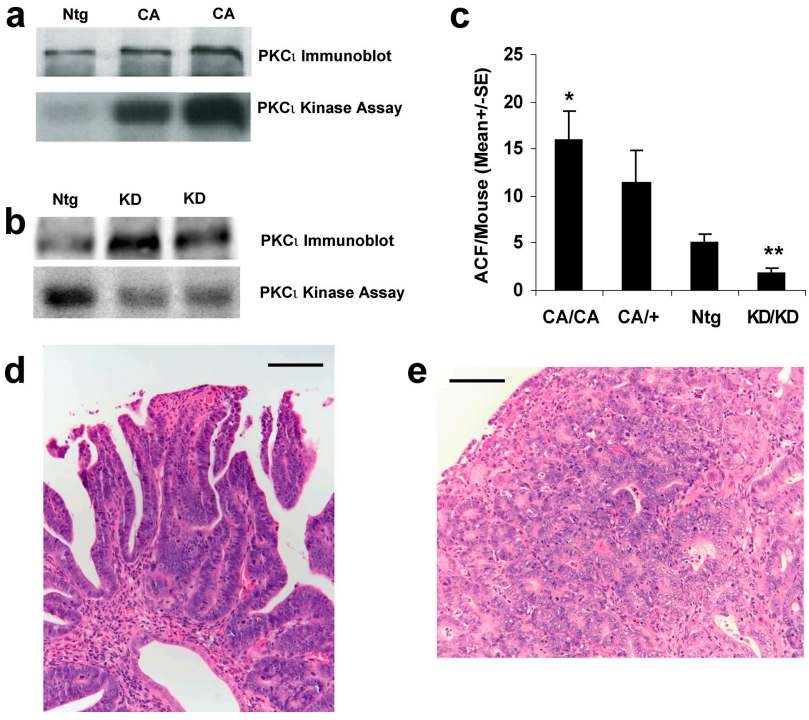

Figure 3. **Transgenic caPKCι mice are highly susceptible to AOM-induced colon carcinogenesis.** (a and b) Total protein lysates from scraped colonic epithelium from nontransgenic (Ntg) and transgenic (a) caPKCι (CA) or (b) kdPKCι (KD) mice were analyzed for PKCι protein (a and b, top) and PKCι activity by immunoprecipitation histone kinase assay (a and b, bottom). (c) Colons from AOM-treated mice were scored for ACF. CA/CA, homozygous caPKCι mice; CA/+, heterozygous caPKCι mice; Ntg, nontransgenic mice; KD/KD, homozygous kdPKCι. Results represent the average ACF/animal ± SEM (n = 4–9; *P = 0.05 vs. Ntg; **P = 0.02 vs. Ntg). (d) H&E-stained section of a tubular adenoma from a nontransgenic mouse. (e) H&E-stained section of a carcinoma in situ from a caPKCι mouse. (d and e) Bars, 100 μm.

ble changes in proliferation or differentiation markers in the colonic epithelium (unpublished data). Next, we treated transgenic caPKCι, transgenic kdPKCι, and nontransgenic mice with AOM to induce colon carcinogenesis (Murray et al., 1999; Gökmen-Polar et al., 2001) and analyzed the mice for preneoplastic lesions, aberrant crypt foci (ACF; Fig. 3 c). Heterozygous transgenic caPKCι mice developed twice as many ACF, and homozygous caPKCι mice developed three times as many ACF, as nontransgenic littermates (Fig. 3 c). In contrast, homozygous transgenic kdPKCι mice developed significantly fewer ACF than nontransgenic mice. Thus, PKCι activity in the colonic epithelium correlates directly with susceptibility to AOM-induced ACF formation.

Next, we assessed the effect of transgenic caPKCι expression on colon tumor formation. Transgenic caPKCι mice exhibited a threefold higher incidence of tumors than nontransgenic mice [63.6% (7/11) vs. 20% (2/10) tumor-bearing mice]. In addition, transgenic caPKCι mice developed predominantly malignant intramucosal carcinomas (6/7 tumors; Fig. 3 e), whereas nontransgenic mice developed mainly benign tubular adenomas (2/3 tumors; Fig. 3 d). Therefore, elevated colonic PKCι activity increases the number of preneoplastic lesions and subsequent colon tumors, and promotes tumor progression from benign adenoma to malignant intramucosal carcinoma. Due to the low tumor incidence in nontransgenic mice it was impractical to assess the effect of kdPKCι on tumor formation.

Given the relationship between PKCι and Ras signaling (Uberall et al., 1999; Coghlan et al., 2000; Kampfer et al., 2001), we assessed whether PKCι is important for Ras-mediated transformation of the intestinal epithelium. We and others (Sheng et al., 2000; Murray et al., 2002; Yu et al., 2003) have used rat intestinal epithelial (RIE) cells to study Ras-mediated transformation, and elucidate the molecular mechanisms by which PKCβII promotes carcinogenesis. Ras-transformed RIE (RIE/Ras) cells were transfected with FLAG-tagged–, wild-type (wt) PKCι or kdPKCι. Both RIE/Ras/wtPKCι and RIE/Ras/kdPKCι cells expressed elevated levels of PKCι when compared with RIE or RIE/Ras cells (Fig. 4 a, top). Immunoblot analysis using an antibody to oncogenic V12 Ras demonstrated that RIE/Ras, RIE/Ras/wtPKCι, and RIE/Ras/kdPKCι cells express comparable levels of oncogenic Ras (Fig. 4 a, second from top). Actin immunoblots confirmed that equal amounts of protein were loaded for each cell line (Fig. 4 a, third from top).

We next assayed RIE, RIE/Ras, RIE/Ras/wtPKCι, and RIE/Ras/kdPKCι cells for total PKCι activity (Jamieson et al., 1999; Fig. 4 a, fourth and fifth from top). Although RIE and RIE/Ras cells expressed equivalent levels of endogenous PKCι (Fig. 4 a, fourth from top), RIE/Ras cells exhibited elevated PKCι activity (Fig. 4 a, fifth from top). Thus, expression of oncogenic Ras leads to activation of endogenous PKCι while having no demonstrable effect on PKCι expression. RIE/Ras/wtPKCι cells expressed elevated PKCι protein and activity when compared with RIE or RIE/Ras cells, whereas RIE/Ras/kdPKCι cells exhibited elevated PKCι protein, but no increase in PKCι activity when compared with RIE/Ras cells (Fig. 4 a, fourth and fifth from top). Immunoprecipitation with an anti-FLAG antibody followed by immunoblot analysis confirmed the expression of FLAG-wtPKCι and FLAG-kdPKCι in RIE/Ras/wtPKCι and RIE/Ras/kdPKCι cells, respectively (Fig. 4 a, second from bottom). Assay of anti-FLAG immunoprecipitates for PKCι activity confirmed that RIE/Ras/wtPKCι cells express catalytically active FLAG-wtPKCι, whereas RIE/Ras/kdPKCι cells express catalytically inac-

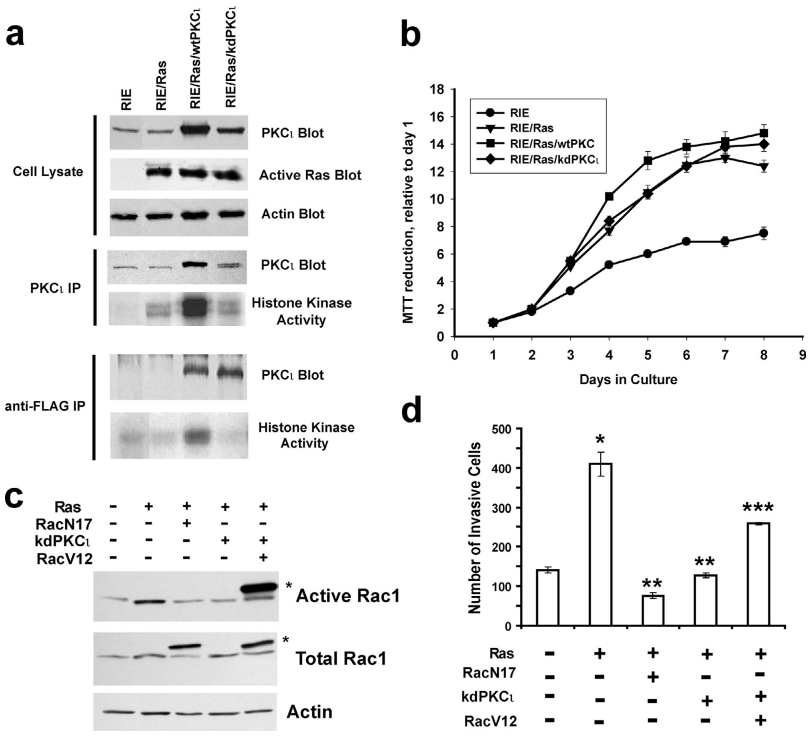

Figure 4. **PKCι is required for oncogenic Ras-induced Rac1 activation and invasion in vitro.** (a) RIE cells were stably transfected with empty vector (RIE), Ras (RIE/Ras), Ras and wtPKCι (RIE/Ras/wtPKCι), or Ras and dnPKCι (RIE/Ras/kdPKCι). Total cell lysates were subjected to immunoblot analysis for PKCι (top), oncogenic V12 Ras (second from top) and β-actin (third from top). Immunoprecipitates using a specific PKCι antibody were analyzed for PKCι expression (fourth from top) and PKCι activity (fifth from top). Anti-FLAG immunoprecipitates were analyzed PKCι expression (second from bottom) and PKCι activity (bottom). (b) Anchorage-dependent growth of RIE cells and RIE cell transfectants. Data represent the mean ± SD from three independent determinations. (c) Active (GTP bound) Rac1 was isolated from the indicated RIE cell transfectants: control empty vector; Ras; Ras and RacN17; Ras and kdPKCι; and Ras and kdPKCι and RacV12. Immunoblot analysis was performed for active Rac1 (top), total cellular Rac1 (middle), and actin (bottom). The asterisk indicates the migration of Myc-tagged, virally expressed Rac1 mutants. (d) The indicated RIE transfectants were assayed for invasion. Data represent the average number of cells invading into the bottom chamber ± SD from three independent experiments. *P = 0.02 versus RIE + control vector; **P < 0.02 versus RIE/Ras; ***P = 0.005 versus RIE/Ras/kdPKCι.

tive FLAG-kdPKCι (Fig. 4 a, bottom). These data demonstrate that oncogenic Ras activates both endogenous and transfected PKCι, and confirm that our kdPKCι construct is deficient in kinase activity.

RIE/Ras cells exhibited an increase in anchorage-dependent growth rate and saturation density compared with RIE cells (Fig. 4 b). Expression of wtPKCι or kdPKCι had little effect on the Ras-mediated increase in growth rate or saturation density (Fig. 4 b). RIE cells expressing wtPKCι or kdPKCι in the absence of oncogenic Ras exhibited no demonstrable change in growth rate compared with RIE cells, and no signs of cellular transformation (unpublished data).

Because Ras transformation is dependent on activation of the small molecular weight GTPase, Rac1 (Qiu et al., 1995), we measured Rac1 activity in RIE/Ras cells (Fig. 4 c). As expected, RIE/Ras cells exhibit elevated Rac1 activity when compared with RIE cells (Fig. 4 c). Expression of either a dominant negative Rac1 (RacN17) mutant (Qiu et al., 1995) or kdPKCι blocked Ras-mediated Rac1 activation. In contrast, expression of a constitutively active Rac1 (RacV12) mutant (Qiu et al., 1995) had little effect on Ras-mediated activation of endogenous Rac1. Expression of wtPKCι in the absence of oncogenic Ras was not sufficient to induce Rac1 activity (unpublished data). Thus, oncogenic Ras activates Rac1 in a PKCι-dependent fashion.

Both Ras and Rac1 have been implicated in cellular motility and invasion (De Corte et al., 2002) and RIE/Ras cells exhibit an invasive phenotype (Fujimoto et al., 2001). Therefore, we assessed whether the invasive phenotype observed in RIE/Ras cells is dependent on Rac1 and PKCι. As expected, RIE/Ras cells are highly invasive, whereas RIE cells are not (Fig. 4 d). Expression of RacN17 or kdPKCι in RIE/Ras cells blocks Ras-mediated invasion (Fig. 4 d). However, expression of RacV12 in RIE/Ras/kdPKCι cells partially restores invasiveness. Thus, oncogenic Ras-mediated cellular invasion is dependent on both Rac1 and PKCι. Interestingly, expression of either wtPKCι or caPKCι in the absence of oncogenic Ras failed to induce invasion, indicating that PKCι is necessary for Ras-mediated invasion, but is not sufficient to induce invasion in the absence of oncogenic Ras (unpublished data).

RIE/Ras cells exhibit anchorage-independent growth in soft agar, whereas RIE cells do not (Fig. 5, a and b). Expression of wtPKCι significantly enhances, and expression of kdPKCι blocks, soft agar growth of RIE/Ras cells (Fig. 5, a and b). Furthermore, expression of RacV12 in RIE/Ras/kdPKCι cells restores soft agar growth (Fig. 5 c). Expression of RacV12 in RIE cells in the absence of oncogenic Ras does not induce soft agar growth, indicating that expression of active Rac1 alone is not sufficient to cause cellular transformation (Fig. 5 c), which is consistent with previous reports that RacV12 exhibits very weak transforming potential (Khosravi-Far et al., 1995). These data demonstrate that PKCι plays a critical role in Ras-mediated transformation of RIE cells because PKCι is required for Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. Our data place PKCι downstream of oncogenic Ras and upstream of Rac1 in a pathway that stimulates invasiveness and soft agar growth, two hallmarks of the transformed phenotype. Next, we assessed the importance of PKCι in

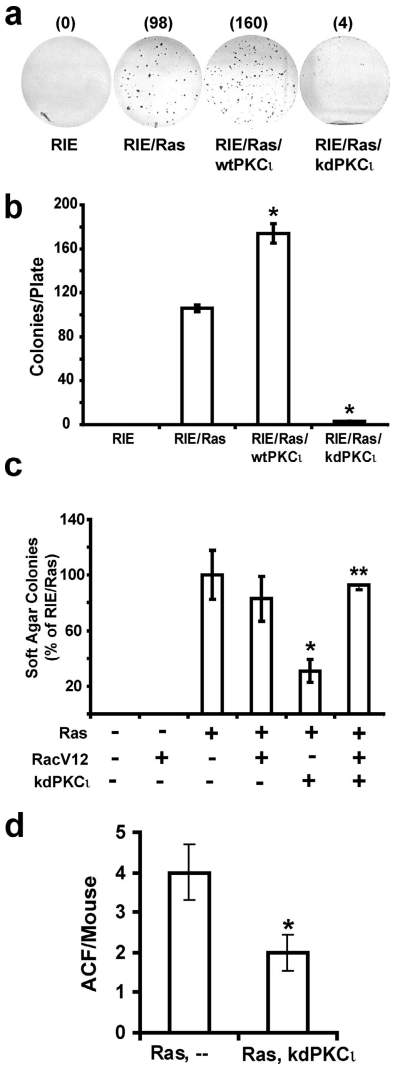

Figure 5. **Expression of dnPKCι blocks Ras-mediated transformation of the intestinal epithelium in vitro and in vivo.** (a and b) RIE cells were stably transfected with control empty vector (RIE), Ras (RIE/Ras), Ras and wtPKCι (RIE/Ras/wtPKCι), or Ras and kdPKCι (RIE/ Ras/ kdPKCι), and evaluated for growth in soft agar. (a) Representative experimental results. Numbers in parentheses represents number of colonies/dish. (b) Values represent the average of three independent experiments ± SEM. *P < 0.002 versus RIE/Ras. (c) The indicated RIE cell transfectants were analyzed as described in panel a. Values represent the average of five determinations ± SEM. *P = 0.008 versus RIE/Ras; **P = 0.0001 versus RIE/Ras/kdPKCι. (d) 12-wk-old K-Ras^{LA2} and K-Ras^{LA2}/kd PKCι mice were analyzed for ACF in the proximal colon. Average number of ACF per mouse is plotted ± the SEM; n = 5; *P = 0.04.

Ras-mediated colon carcinogenesis in vivo using transgenic mice expressing a latent oncogenic K-ras allele (G12D) that is activated by spontaneous recombination (Johnson et al., 2001). Latent K-ras (K-Ras^{LA2}) mice develop Ras-dependent lung carcinomas and colonic ACF (Johnson et al., 2001). We bred our transgenic kdPKCι mice with K-Ras^{LA2} mice to generate bitransgenic K-Ras^{LA2}/kdPKCι mice, and assessed them for spontaneous ACF development (Fig. 5 d). K-Ras^{LA2}/kdPKCι mice developed significantly fewer ACF in the proximal colon than K-Ras^{LA2} mice. These data are consistent with our results in RIE/Ras cells in vitro, and demon-

strate that PKCι is critical for oncogenic K-ras–mediated colon carcinogenesis in vivo.

Our results provide direct evidence that PKCι and Rac1 are necessary for the transformed phenotype induced by oncogenic Ras. Rac1 has been shown to be required for transformation by both H-Ras and K-Ras, the two most commonly mutated forms of Ras in human cancers. Our data demonstrate that PKCι is also required for both H-Ras– and K-Ras–mediated transformation. Although H-Ras and K-Ras have both common and distinct effectors, both of these Ras isoforms activate Rac1, though K-Ras appears more effective than H-Ras (Walsh and Bar-Sagi, 2001). We have shown that H-Ras induces Rac1 activity through a PKCι-dependent pathway and that PKCι is required for K-Ras–mediated colon carcinogenesis. Given the increased propensity of K-Ras to activate Rac1, it is likely that the Ras→PKCι→Rac1 pathway we have elucidated in RIE cells is also important for K-Ras–mediated colon carcinogenesis in vivo. Interestingly, PKCι and Rac1 have also been implicated in epithelial cell polarity through formation of complexes containing PKCι, Par6, and Rac1 (Noda et al., 2001). Rac1 is thought to regulate PKCι activity within these complexes to affect cell polarity (Noda et al., 2001). Our data now implicate signaling through PKCι–Par6–Rac1 complexes in Ras-mediated transformation.

In this report, we present conclusive evidence that PKCι is critical for colonic epithelial cell transformation both in vitro and in vivo. Interestingly, disruption of PKCι signaling by kdPKCι has little effect on normal intestinal epithelial cell homeostasis in vitro and in vivo, suggesting that PKCι may be an attractive target for development of novel therapeutics against colon cancer.

Materials and methods

Analysis of PKCι expression in mouse and human colon tumors

AOM-induced mouse colon tumors were produced in C57Bl/6 mice as described previously (Gökmen-Polar et al., 2001). Fresh frozen tissue from human colon carcinomas and uninvolved colonic epithelium was obtained from surgical specimens. Isolation of RNA and protein for RT-PCR and immunoblot analysis, respectively, was performed as described previously (Gökmen-Polar et al., 2001). Immunoblot analysis for PKCι and actin was conducted using isozyme-specific antibody against PKCι and actin (Santa Cruz Biotechnology, Inc.) as described previously (Murray and Fields, 1997; Gökmen-Polar et al., 2001). We determined previously that this PKCι antibody recognizes PKCι but not PKCζ (Murray and Fields, 1997). Primers for RT-PCR analysis were as follows: PKCι forward primer, 5′-GCTTATGTTTGAGATGATGGCGG-3′, and PKCι reverse primer, 5′-GTGACAACCCAATCGTTCCG-3′; and actin forward primer, 5′-GTGGGCCGCTCTAGGCACCAA-3′, and actin reverse primer, 5′-CTCTTTGATGTCACGCACGATTTC-3′. Colon tumors and uninvolved colonic epithelium from AOM-treated mice were fixed in 10% buffered formalin, sectioned, and subjected to antigen retrieval (Vector Laboratories). Immunohistochemical detection of PKCι was performed using the specific PKCι antibody (Santa Cruz Biotechnology, Inc.) and the DAKO LSAB2 (DAB) detection system (DakoCytomation). Specificity of immunostaining for PKCι was demonstrated by inclusion of a fivefold molar excess of the peptide used to generate the PKCι antibody (Santa Cruz Biotechnology, Inc.) in the antibody dilution. Digital images were acquired on a microscope (model DX51; Olympus) equipped with a DP70 digital camera using a 20× objective lens. Images were captured using the DP Controller software and processed in Adobe Photoshop.

Production of transgenic mice and carcinogenesis studies

Human caPKCι and kdPKCι cDNAs were generated and characterized previously (Jamieson et al., 1999; Lu et al., 2001). Transgenic caPKCι and kdPKCι mice were generated on a C57Bl/6 background using the *Fabpl*$^{4x\ at\ -132}$ promoter (Simon et al., 1997; provided by J. Gordon, Washington University, St. Louis, MO) to direct transgene expression to the colonic epithelium (Murray et al., 1999). Isolation of colonic epithelium, immunoblot analysis for PKCι, and immunoprecipitation histone kinase assays were described previously (Jamieson et al., 1999; Murray et al., 1999). Transgenic caPKCι, transgenic kdPKCι, and nontransgenic mice were injected with either 10 mg/kg AOM or saline as described previously (Gökmen-Polar et al., 2001). ACF analysis was performed 12 wk after the last AOM injection (Murray et al., 2002) using well-defined criteria (McLellan et al., 1991). Mice were analyzed at 40 wk for tumor number, size, location, and pathological grade as described previously (Gökmen-Polar et al., 2001). All tumors were classified as either tubular adenomas or intramucosal carcinomas (carcinoma in situ) by Z. Gatalica, a board-certified pathologist. Digital images of the tumors were captured on a microscope (model Eclipse E600; Nikon) equipped with a ProgRes C14 camera (Jenoptik) using a 20× objective lens. Images were acquired using ProgRes C14 software with Microsoft Photoeditor and processed with Microsoft Photoshop.

Transgenic K-ras^{LA2} mice (Johnson et al., 2001; provided by T. Jacks, Massachusetts Institute of Technology, Cambridge, MA) were bred to transgenic kdPKCι mice to obtain bitransgenic K-ras^{LA2}/kdPKCι mice. At 12 wk old, transgenic K-ras^{LA2} and bitransgenic K-ras^{LA2}/kdPKCι mice were assessed for spontaneous ACF formation (McLellan et al., 1991; Murray et al., 1999).

RIE cell transfections and cellular analyses

RIE cells and derivatives were grown in DME containing 5% FBS as described previously (Ko et al., 1998). RIE/Ras cells were described elsewhere (Sheng et al., 2000; provided by H.M. Sheng, University of Texas Medical Branch [UTMB], Galveston, TX). Microarray analysis of RIE/Ras cells demonstrated that these cells do not express PKCζ (unpublished data). Human wtPKCι and kdPKCι cDNAs were cloned into the pBABE/FLAG/puro retroviral expression vector and virus stocks were produced using Phoenix-E cells (provided by G. Nolan, Stanford University, Palo Alto, CA). Puromycin-resistant, stable transfectants were generated as described at http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html. Expression of FLAG-epitope–tagged PKCι was confirmed by immunoblot analysis using anti-FLAG antibody (Sigma-Aldrich), and PKCι kinase activity was determined by immunoprecipitation histone kinase assay as described previously (Jamieson et al., 1999).

Recombinant retroviruses containing Myc-tagged RacN17 or Myc-tagged RacV12 were generated by excising the Myc-tagged Rac1 constructs from pEXV/Rac vectors (Qiu et al., 1995) with EcoRI and ligating them into the EcoRI site of the LZRS-GFP retrovirus. The entire coding sequence of each construct was confirmed by DNA sequence analysis. LZRS-GFP-Rac1 retroviruses were used to infect RIE cells and derivative cell lines using a protocol described previously (Ireton et al., 2002). Rac1 activity was assessed by affinity isolation of GTP-bound Rac1 using a protocol described previously (Sander et al., 1998). Active GTP-bound Rac1 and total Rac1 were identified by immunoblot analysis using a Rac1 mAb (BD Biosciences) and quantitated by densitometry.

Invasiveness of RIE cell transfectants was assessed in Transwell inserts precoated with Matrigel (6.5-mm diam, 8-μm pore size; BD Biosciences). DME containing 10% FBS was added to the bottom chamber and 5×10^4 cells were suspended in 500 μl of serum-free DME and placed in the top chamber of the Transwell insert. Cells were incubated for 22 h at 37°C in 5% CO_2, at which time noninvading cells were removed from the top chamber. Cells that had invaded through the Matrigel-coated filter were fixed in 100% methanol, stained with crystal violet, and counted on a microscope (Nikon) using a calibrated ocular grid. 15 representative areas of the bottom chamber were counted to determine the number of invasive cells in each well.

To assess anchorage-independent growth, RIE cell transfectants were suspended in DME supplemented with 10% FBS, 1.5% agarose, and a 1% insulin, transferrin, and selenium solution (Sigma-Aldrich), and plated (300 cells/60-mm dish) on a layer of 1.5% agar containing the same medium. Cell colonies were fixed with 20% methanol and stained with Giemsa after 7–14 d in culture and quantified under a dissecting microscope (Nikon).

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