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K-Ras-mediated Increase in Cyclooxygenase 2 mRNA Stability Involves Activation of the Protein Kinase B[1]

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ABSTRACT

Cyclooxygenase (COX) 2 expression is regulated via the Ras signaling pathway, and induction of mutated *Ras* rapidly increases COX-2 levels in intestinal epithelial cells. Protein kinase B (Akt/PKB) is an important effector of Ras signaling and a critical component of Ras-mediated transformation. Here we investigate the role of Akt/PKB in K-Ras-mediated induction of COX-2. Rat intestinal epithelial cells (IEC-6) were transfected with an inducible K-Ras^{Val12} cDNA (IEC-iK-Ras cells). Addition of 5 mM isopropyl-1-thio-β-D-galactopyranoside induced the expression of K-Ras^{Val12}, followed by increased activity of extracellular signal-regulated kinase and Akt/PKB. COX-2 levels were dramatically increased after induction of K-Ras^{Val12}. Inhibition of MAPK/ERK kinase activity by PD 98059 completely blocked the K-Ras-mediated induction of COX-2, whereas inhibition of PI3K/Akt/PKB activity with LY 294002 or by expressing a dominant negative Akt (Akt-K179M) partially blocked the induction of COX-2 by K-Ras. Transient transfection of cells with phosphatidylinositol 3-kinase and Akt expression vectors revealed that PI3/Akt/PKB activity predominantly regulates the stability of COX-2 mRNA. Thus, Akt/PKB activity is involved in K-Ras-induced expression of COX-2 and stabilization of COX-2 mRNA largely depends on the activation of Akt/PKB.

INTRODUCTION

Ras mutations are found in a wide variety of human malignancies and in ~50% of colorectal carcinomas (1). Oncogenic mutations in *Ras* result in activation of downstream signaling proteins including Raf/MEK/ERKs[3] (2, 3), Raf-independent signaling proteins that belong to the Rho family (4, 5), and the PI3K/Akt/PKB pathway (6, 7). A specific subset of genes is subsequently modulated, which results in oncogenic Ras transformation (8–10).

Prostaglandin endoperoxide synthase-2 (*Ptgs-2*), commonly referred to as cyclooxygenase-2 (*COX-2*), is a target of the Ras signaling pathway. Expression of mutated Ha-Ras results in morphological transformation associated with rapid induction of COX-2 in fibroblasts (11) and intestinal epithelial cells (10). The induction of COX-2 expression by Ras involves both transcriptional and posttranscriptional regulation. Although the precise role of COX-2 in Ras-mediated transformation is not clear, evidence is mounting to indicate that COX-2 expression provides a growth and survival advantage to intestinal epithelial cells (12–14).

The serine/threonine kinase Akt (or Akt/PKB) is a direct downstream effector of PI3K (15, 16). Akt/PKB can be modulated by multiple intracellular signaling pathways and acts as a transducer for many pathways initiated by growth factor receptors that activate PI3K (reviewed in Ref. 17). Akt/PKB regulates gene transcription by directly or indirectly modifying phosphorylation of transcription factors (18–24). Activation of the PI3K/Akt/PKB pathway is important in Ras transformation of mammalian cells and essential for Ras-induced cytoskeletal reorganization (6). The PI3K/Akt/PKB signaling pathway plays a critical role in R-Ras-mediated transformation, adhesion, and cell survival (7). Evidence suggests that the PI3K/Akt/PKB pathway promotes growth factor-mediated cell survival and inhibits apoptosis (25) by modifying the antiapoptotic and proapoptotic activities of members of the *Bcl-2* gene family (26, 27). These observations strongly suggest that the PI3K/Akt/PKB pathway is oncogenic and involved in the neoplastic transformation of mammalian cells.

In the present study, we sought to elucidate the role of Akt/PKB in K-Ras-mediated induction of COX-2 in intestinal epithelial cells. Our results indicate that expression of oncogenic K-Ras activates the Raf/MEK/ERK and PI3K/Akt/PKB pathways. Both MEK/ERK and Akt/PKB activities are required for K-Ras-mediated induction of COX-2. The activation of MEK is essential for both increased transcription and stability of COX-2 mRNA, whereas Akt/PKB activity is largely responsible for the stabilization of COX-2 mRNA.

MATERIALS AND METHODS

Cell Culture. Rat intestinal epithelial cells (IEC-6) were obtained from ATCC (Rockville, MD). An IEC-iK-Ras cell line with an inducible activated K-Ras^{Val12} cDNA was generated by using the LacSwitch eukaryotic expression system (Stratagene, La Jolla, CA). The cells were maintained in DMEM containing 10% fetal bovine serum, 400 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD), and 150 μg/ml Hygromycin B (Calbiochem, San Diego, CA). The K-Ras^{Val12} cDNA is under the transcriptional control of the Lac operon. IPTG (Life Technologies, Inc.) at a concentration of 5 mM was used to induce the expression of mutated K-Ras. PD 98059, LY294002, and AG1478 were purchased from Calbiochem.

Northern Blot Analysis. For determination of mRNA stability, IEC-iK-Ras cells were treated with vehicle or IPTG for 48 h, and then the transcription was stopped by addition of 100 μM of DRB (Sigma Chemical Co., St. Louis, MO). RNA samples were extracted, separated on formaldehyde-agarose gels, and blotted on to nitrocellulose membranes as previously described (11). The blots were hybridized with cDNA probes labeled with [α-^{32}P]dCTP by random primer extension (Stratagene) and then subjected to autoradiography. rRNA signals at 18S were used as controls to determine integrity of RNA and equality of the loading.

Immunoblot Analysis and Antibodies. Immunoblot analysis was performed as previously described (28). Cells were lysed for 30 min in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethyl-sulfonyl fluoride, 10 μg/ml aprotinin, 1 mM sodium orthovanadate). Cell lysates were denatured and fractionated by SDS-PAGE, and after electrophoresis the proteins were transferred to nitrocellulose membranes. The filters were then probed with the indicated antibodies, developed by the enhanced chemiluminescence system (ECL; Amersham, Arlington Heights, IL). The anti-pan Ras antibody was purchased from Calbiochem (La Jolla, CA). The anti-COX-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antiphosphorylated (serine 473) Akt antibody was obtained from New England Biolabs (Beverly, MA) and the antiactive ERK1/2 antibody was from Promega (Madison, WI). The anti-β-actin antibody was purchased from Sigma.

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[3] The abbreviations used are: MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; Akt/PKB, protein kinase B; COX, cyclooxygenase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; DRB, 5,6-dichlorobenzimidazole riboside; 3′ UTR, 3′ untranslated region; PI3K, phosphatidylinositol 3-kinase; CMV, cytomegalovirus; IEC, intestinal epithelial cell; HA, hyaluronic acid.

Ectopic Expression of Akt. To establish the IEC-iK-Ras/Akt-K179M cell line, stable transfection was performed using Lipofectin (Life Technologies, Inc.). A 1.5-kb *Hin*dIII-*Bam*HI fragment containing the HA-tagged dominant negative Akt-K179M cDNA (a gift from Dr. Philip N. Tsichlis, Thomas Jefferson University, Philadelphia, PA) was ligated into the eukaryotic expression vector pZeoSV2(+) (Invitrogen, Carlsbad, CA). The resultant pZeoSV2/Akt-K179M vector was then transfected into the IEC-iK-Ras cells and selected in DMEM containing hygromycin, neomycin, and zeocin (250 μg/ml) to generate the IEC-iK-Ras/Akt-K179M clones.

ERK Kinase Assay. p42/44 MAP kinase activity was measured by determining the transfer of the phosphate group of ATP to a peptide that is a highly specific substrate for p42/44 MAP kinase according to the manufacturer's instructions (BIOTRAK system; Amersham).

Akt Assay. For determination of Akt kinase activity we used the Akt kinase assay kit produced by New England Biolabs, according to the manufacturer's instructions. IEC-iK-Ras cells were treated with IPTG and then lysed at the indicated times. Akt was immunoprecipitated using a monospecific Akt antibody. The immunoprecipitate was then incubated with a GSK-3 fusion protein in the presence of ATP. Phosphorylation of GSK-3 was measured by Western blotting using an anti-phospho-GSK-3α/β (Ser21/9) antibody.

Transfection of Reporter Constructs. The assays to determine activity of the COX-2 promoter and stability of COX-2 3′ UTR are described elsewhere (10). To achieve stable transfection, a reporter construct containing the 5′-flanking region of the human *COX-2* gene (phPES2−1432/+59, a gift from Dr. Hiroyasu Inoue, National Cardiovascular Center Research Institute, Osaka, Japan; Ref. 29) or COX-2 3′ UTR (pcDNA3/Luc+3′ UTR, a gift from Drs. Dan Dixon and Stephen Prescott, University of Utah, Salt Lake City; Ref. 10, 30) was transfected into IEC-iK-Ras cells, which are referred to as IEC-iK-Ras/COX-2 5′-luc or IEC-iK-Ras/luc-COX-2 3′ UTR cells. Transfected cells were selected by growth in media containing neomycin (600 μg/ml), hygromycin (150 μg/ml), and zeocin (250 μg/ml). Pooled clones were evaluated for luciferase activity. Firefly luciferase values were standardized to the protein concentration, and the data are presented as mean ± SE of assays performed in quadruplicate.

For transient transfections, cells were cotransfected with 0.5 μg of one of the COX-2 firefly luciferase constructs (phPES2−1432/+59 or pcDNA3/Luc+3′ UTR) and 1 ng of the pRL-CMV plasmid containing the CMV immediate-early enhancer/promoter region upstream of the renilla luciferase gene (Promega) along with 0.5 μg of Akt expression vectors (myristylated form of Akt-myr or Akt-K179M cDNA, gifts from Dr. Philip N. Tsichlis) or pSG5-Δp85 (a gift from Dr. Bart Vanhaesebroeck, Ludwig Institute for Cancer Research, London, United Kingdom). Transfected cells were cultured for 24 h and then lysed in lysis buffer (Promega). Twenty μl of lysate were used for both the firefly and renilla luciferase readings, which were measured using a Dual-Luciferase Reporter assay system (Promega). Firefly luciferase values were standardized to renilla values.

RESULTS

Establishment of IEC-iK-Ras Cells. Mutations of *K-Ras* occur during neoplastic transformation in several different solid malignancies, including ~50% of colorectal carcinomas (1). To investigate the phenotypic alterations that result from K-Ras-mediated transformation, a conditionally transformed IEC line was established, in which expression of mutated K-Ras$^{\text{Val12}}$ can be induced (referred to here as IEC-iK-Ras). IEC-iK-Ras cells displayed a nontransformed morphology similar to parental IEC-6 cells when grown in normal medium (Fig. 1*A*). Treatment of cells with 5 mM IPTG induced the expression of mutated K-Ras. The levels of Ras protein increased slowly up to 12 h and reached a peak at 48 h after addition of IPTG (Fig. 1*B*). Morphological transformation of the IEC-iK-Ras cells was observed between 48 and 72 h after initiation of IPTG treatment. During this interval, cell-cell contact inhibition was lost, and the cells acquired a spindly appearance, growing in overlapping clusters. Both IEC-6 and uninduced IEC-iK-Ras cells were unable to grow in an anchorage-independent fashion. However, in the presence of IPTG, IEC-iK-Ras cells rapidly formed colonies in soft agarose (Fig. 1*C*).

Induction of COX-2 by K-Ras$^{\text{Val12}}$. The presence of oncogenic *Ras* is known to induce the expression of COX-2 (10, 11, 28, 31). In IEC-iK-Ras cells, COX-2 was expressed at low levels before IPTG treatment, but COX-2 protein was markedly elevated 24 h after addition of IPTG to the cell culture medium (Fig. 2*A*). To study the mechanisms underlying the induction of COX-2 by K-Ras, we stably transfected the luciferase reporter gene linked with the *COX-2* promoter region into IEC-iK-Ras cells (IEC-iK-Ras/COX-2 5′-Luc). The 5′-flanking region of the human *COX-2* gene (nucleotides −1432 to +59) exhibited promoter activity that was increased by induction of oncogenic K-Ras. Treatment with IPTG for 24 h increased *COX-2* promoter activity by ~70% (Fig. 2*B*).

To determine whether induction of K-Ras affected the stability of COX-2 mRNA, the rate of COX-2 mRNA degradation was determined by Northern blot analysis. As demonstrated in Fig. 2*C*, COX-2 mRNA was rapidly degraded in noninduced IEC-iK-Ras cells ($T_{1/2}$ ~ 30 min). IPTG treatment increased the stability of COX-2 mRNA and

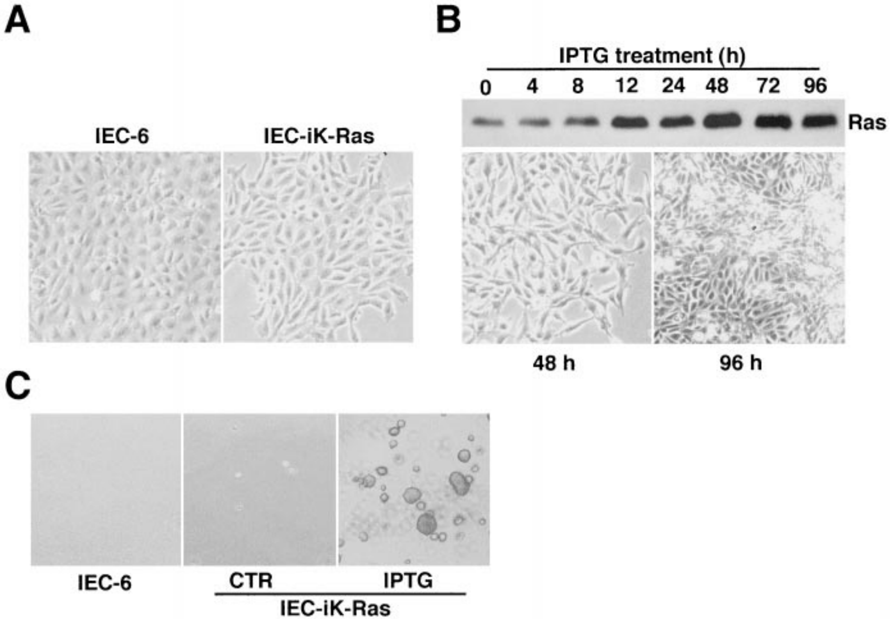

Fig. 1. Conditional transformation of IEC-iK-Ras cells. *A*, morphology of IEC-6 cells and IEC-iK-Ras cells (original magnification, ×100). *B*, induction of K-Ras and transformation of IEC-iK-Ras cells. IEC-iK-Ras cells were treated with 5 mM IPTG and lysed in radioimmunoprecipitation assay buffer at the indicated time points for Western analysis. For evaluation of cell morphology, IEC-iK-Ras cells were treated with 5 mM IPTG for the indicated times and photographed (×100). *C*, anchorage-independent growth of IEC-iK-Ras cells. 1×10^4 cells were mixed with Sea plaque agarose at a final concentration of 0.4% in DMEM medium containing vehicle (CTR) or IPTG. The plates were incubated for 10 days. Colonies were photographed using a camera attached to an inverted microscope (×40).

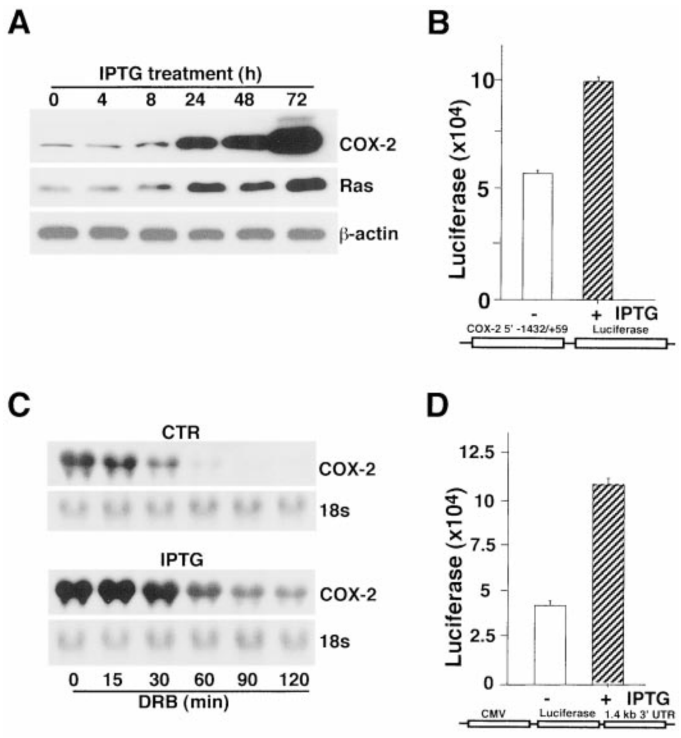

Fig. 2. Regulation of COX-2 expression by K-Ras. *A,* induction of COX-2 by K-Ras. IEC-iK-Ras cells were treated with IPTG and cell lysates were collected at the indicated time points. The levels of COX-2 protein were determined by Western blot analysis. For loading controls, β-actin levels are shown in all Western blot analyses. *B,* transfection of reporter vector under the transcriptional control of *COX-2* promoter (−1432/+59). IEC-iK-Ras cells were cotransfected with phPES2 (−1432/+59) containing the 5′-flanking region of the human *COX-2* gene and pcDNA3/zeo. Stable transfectants were selected by addition of 300 μg/ml of zeocin. Cells were plated in 24-well plates and treated with or without 5 mM IPTG for 24 h. Twenty μl of lysate was used for the firefly luciferase readings that are plotted as the mean ± SE of assays performed in quadruplicate. The results shown are representative of three separate experiments. *C,* degradation of COX-2 mRNA. IEC-iK-Ras cells were treated with vehicle or IPTG for 48 h. Then transcription was stopped by addition of 100 μM DRB. The RNA samples were isolated at the indicated time points after DRB treatment and the levels of COX-2 mRNA were determined by Northern blot analysis. *D,* stable transfection of reporter constructs linked with COX-2 3′ UTR. IEC-iK-Ras cells were transfected with pcDNA3/Luc+3′ UTR. Stable transfectants were selected for luciferase assays. The firefly luciferase readings are plotted as the mean ± SE of assays performed in quadruplicate. The results were similar in three separate experiments.

extended the $T_{1/2}$ up to ~ 90 min. We next prepared IEC-iK-Ras cells that contained the CMV promoter-driven luciferase reporter gene linked with 1.4 kb of the COX-2 3′ UTR (IEC-iK-Ras/luc+COX-2 3′UTR). Treatment with IPTG for 24 h increased the luciferase activity by >100% in IEC-iK-Ras/luc+COX-2 3′UTR cells, suggesting that oncogenic K-Ras stabilized the 3′ UTR of COX-2 mRNA (Fig. 2*D*).

Induction of ERK and Akt/PKB by K-Ras. Raf/MEK/ERKs represent an important downstream signaling pathway of Ras. As expected, induction of K-Ras increased the levels of activated ERK1/2 (phosphorylated ERK1/2) in IEC-iK-Ras cells (Fig. 3*A*). Kinase activity assays revealed that expression of K-Ras$^{\text{Val12}}$ greatly increased the ERK kinase activity (Fig. 3*B*). Akt/PKB can be activated in a Ras-dependent or -independent manner (17). To determine whether expression of K-Ras$^{\text{Val12}}$ activates Akt/PKB, we first measured the levels of active Akt in Ras-induced IEC-iK-Ras cells. Western blot analysis showed that induction of K-Ras$^{\text{Val12}}$ increased the levels of the activated form of Akt/PKB (phosphorylated at serine 473) in IEC-iK-Ras cells (Fig. 3*A*). The expression of oncogenic K-Ras significantly elevated the levels of Akt kinase activity in IEC-iK-Ras cells, as determined by its capability to phosphorylate GSK-3 kinase (Fig. 3*C*).

Regulation of COX-2 by ERK and Akt/PKB. To determine the mechanism by which K-Ras induces the expression of COX-2, we evaluated the role of the Raf/MEK/ERK and PI3K/Akt/PKB pathways in the regulation of COX-2. As demonstrated in Fig. 4*A*, treatment with IPTG strongly induced the expression of COX-2 in IEC-iK-Ras cells compared with controls. Addition of the selective MEK inhibitor, PD 98059 (50 μM), completely blocked the K-Ras-mediated induction of COX-2, whereas addition of the selective PI3K inhibitor, LY 294002 (20 μM), partially inhibited K-Ras-mediated COX-2 induction. To determine the interaction between the Raf/MEK/ERK and PI3K/Akt/PKB pathways, K-Ras$^{\text{Val12}}$ was induced in the presence of PD 98059 or LY 294002. IPTG treatment for 24 h increased the levels of active pERK1/2, pAkt, and COX-2 (Fig. 4*B*). Addition of PD 98059 abolished the Ras-mediated induction of pERK1/2 and blocked induction of pAkt and COX-2, whereas LY 294002 blocked the elevation of pAkt and partially inhibited the induction of COX-2 but did not affect the levels of active pERK1/2. A specific inhibitor of the epidermal growth factor receptor signal transduction pathway, AG1478 (25 μM), did not alter the levels of K-Ras-induced pERK1/2, pAkt, or COX-2.

To further confirm the role of Akt/PKB in K-Ras-mediated induc-

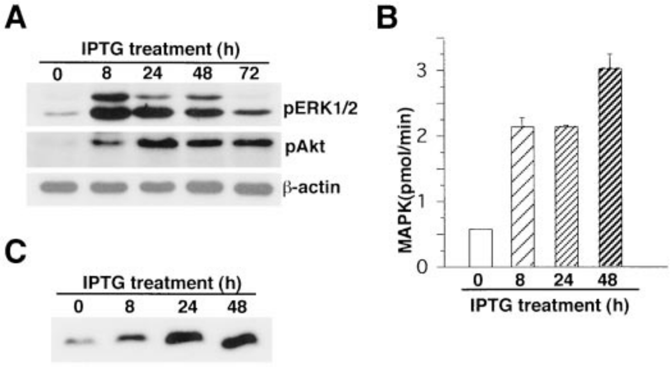

Fig. 3. The activation of MEK/ERK and Akt/PKB pathways by K-Ras. *A,*K-Ras induction of ERKs and Akt/PKB. IEC-iK-Ras cells were treated with IPTG and lysed at the time points indicated. Phosphorylated ERK1/2 and phosphorylated Akt were determined by Western blot analysis. These experiments were repeated three times. *B,* ERK kinase assay. IEC-iK-Ras cells were treated with IPTG and lysed at the indicated time points. ERK1/2 kinase activity was determined using BIOTRAK system. *C,* Akt kinase assay. IEC-iK-Ras cells were treated with IPTG and lysed at indicated time points. Akt was immunoprecipitated using a monospecific Akt antibody. The immunoprecipitate was then incubated with a GSK-3 fusion protein in the presence of ATP. Phosphorylation of GSK-3 was measured by Western blotting using an anti-phospho-GSK-3α/β (Ser21/9) antibody. The results were similar in three independent experiments.

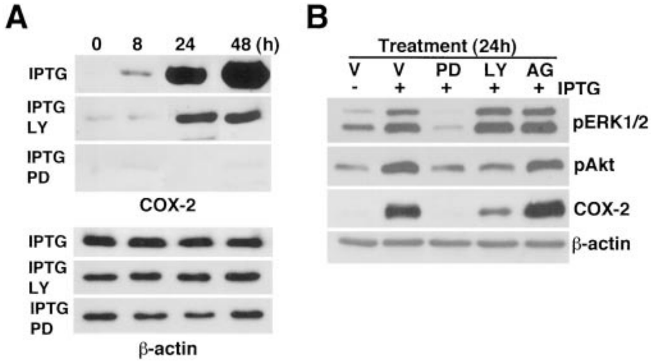

Fig. 4. Inhibition of K-Ras-induced COX-2 by PD 98059 and LY 294002. *A,* IEC-iK-Ras cells were treated with IPTG, IPTG plus LY 294002, or IPTG plus PD 98059 for the indicated times and COX-2 protein levels were determined by Western blotting analysis. *B,* IEC-iK-Ras cells were treated with IPTG in the presence of DMSO (V), PD 98059 (50 μM), LY 294002 (20 μM), or AG 1478 (25 μM) for 24 h. The levels of pERK1/2, pAkt, and COX-2 were determined by Western blot analysis.

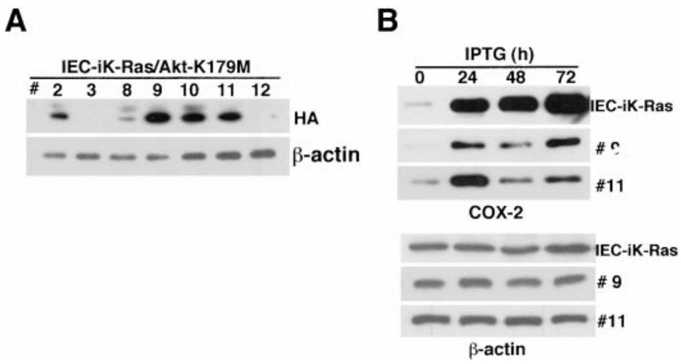

Fig. 5. Inhibition of K-Ras-induced COX-2 by Akt-K179M. *A,* establishment of IEC-iK-Ras/Akt-K179M cells. A pZeoSV2/Akt-K179M vector was transfected into the IEC-iK-Ras cells and positive clones were selected by growth in DMEM containing hygromycin, neomycin, and zeocin (250 μg/ml). The expression of HA in IEC-iK-Ras/Akt-K179M positive clones was determined by Western blot analysis. *B,* COX-2 expression in IEC-iK-Ras/Akt-K179M cells. IEC-iK-Ras, or IEC-iK-Ras/Akt-K179M clone 9 and 11 were treated with IPTG for the indicated times. The COX-2 protein levels were determined by Western blot analysis.

tion of COX-2, IEC-iK-Ras cells were transfected with an expression vector containing a HA-tagged dominant negative form of Akt (Akt-K179M). Stably transfected clones 9 and 11, which expressed high levels of HA, were selected and are referred to as IEC-iK-Ras/Akt-K179M (Fig. 5*A*). In agreement with the effect of LY 294002 on COX-2 expression, ectopic expression of Akt-K179M significantly (but incompletely) blocked the K-Ras-mediated induction of COX-2 (Fig. 5*B*).

Transcriptional and Posttranscriptional Regulation of COX-2. As demonstrated in Fig. 2, oncogenic K-Ras regulates the expression of COX-2 at both transcriptional and posttranscriptional levels. It was of interest to determine the signaling pathway(s) responsible for the regulation of COX-2 expression at both levels. IEC-iK-Ras/COX-2 5′-Luc and IEC-iK-Ras/luc-COX-2 3′ UTR cells were treated with IPTG in the presence or absence of PD 98059 (50 μM). Luciferase assays of extracts from these cells revealed that PD 98059 almost completely blocked the K-Ras-induced transcription of *COX-2* (Fig. 6*A*) and K-Ras-mediated stabilization of COX-2 mRNA (Fig. 6*B*), indicating that MEK/ERK activity is required for both transcriptional and posttranscriptional modulation of COX-2 expression.

To determine the role of Akt/PKB in the regulation of COX-2, we transiently cotransfected IEC-iK-Ras cells with COX-2 reporter vectors and Akt expression vectors. As demonstrated in Fig. 7*A*, expression of active Akt-myr or dominant negative Akt-K179M did not significantly alter the activity of the *COX-2* promoter in both Ras-induced and uninduced IEC-iK-Ras cells. However, expression of Akt-K179M reduced the stability of the COX-2 3′ UTR by 35% in uninduced IEC-iK-Ras cells and blocked the K-Ras-induced stabilization of COX-2 3′ UTR. Ectopic expression of constitutively active Akt-myr increased the stability of the COX-2 3′ UTR by 110%, so that the induction of K-Ras^{Val12} only slightly increased the stability of COX-2 3′ UTR.

Akt/PKB is a direct downstream effector of PI3K, and its activation often depends on PI3K activity. We next investigated the role of PI3K in the regulation of COX-2 by cotransfecting IEC-iK-Ras cells with COX-2 reporter vectors and a PI3K expression vector. As demonstrated in Fig. 7*C*, expression of the dominant negative regulatory subunit of PI3K (Δp85) did not alter the K-Ras-induced activity of the *COX-2* promoter (Fig. 7*C*) but completely inhibited the K-Ras-mediated stabilization of the COX-2 3′ UTR (Fig. 7*D*).

DISCUSSION

Numerous studies indicate that cyclooxygenase activity and prostaglandin synthesis may be involved in promoting intestinal carcinogenesis. Evidence is mounting to suggest that COX-2 expression in colorectal carcinoma cells provides a growth and survival advantage (13, 14). Although the precise role of COX-2 in Ras transformation is not understood completely, the induction of COX-2 by activation of Ras is well documented (10, 11, 28, 31). In the present study, we demonstrate that expression of mutated K-Ras^{Val12} results in transformation of intestinal epithelial cells. In agreement with the observations in Ha-Ras-transformed cells, K-Ras also induces COX-2 expression, preceding the morphological transformation, suggesting that *COX-2* is one possible target gene of oncogenic Ras.

It is well documented that both Ras/Rac1/MEKK1/JNK and Ras/Raf-1/MEK/ERK signal transduction pathways are necessary for the transcriptional induction of *COX-2*. Ras activates the MEKK1/JNKK/JNK kinase cascade (4, 32), leading to phosphorylation of c-Jun, which results in transcriptional activation of *COX-2* via the cyclic AMP response element (CRE; 33, 34). Inhibition of MEK/ERK activity leads to a reduction in *COX-2* transcription (33). Subbaramaiah *et al.* (35) reported that inhibition of MEK, JNK, and p38 MAPK blocked the induction of COX-2 by ceramide and that phosphorylation of c-Jun and transactivation via the CRE cis element in the *COX-2* promoter is required for the induction of COX-2 by ceramide. The CCAAT/enhancer-binding protein β (C/EBP β) is thought to be required for COX-2 induction via the Raf/MEK/ERK pathway (34). Our results show that MEK/ERK activity is essential for the K-Ras-mediated induction of COX-2 and that treatment with PD 98059 blocks K-Ras-induced transcriptional activation of the *COX-2* promoter.

Cumulative evidence indicates that the expression of COX-2 is also regulated at the posttranscriptional level (36). We recently reported that the induction of COX-2 in conditionally Ha-Ras^{Val12} transformed Rat-1 cells occurs via a modest increase in *COX-2* transcription with a significant increase in the stability of COX-2 mRNA (11). Induction of oncogenic Ras stabilizes the 3′ UTR of COX-2 mRNA in intestinal epithelial cells. A conserved A-U rich region (ARE) is responsible for the rapid turnover of COX-2 mRNA (30) and for the stabilization of

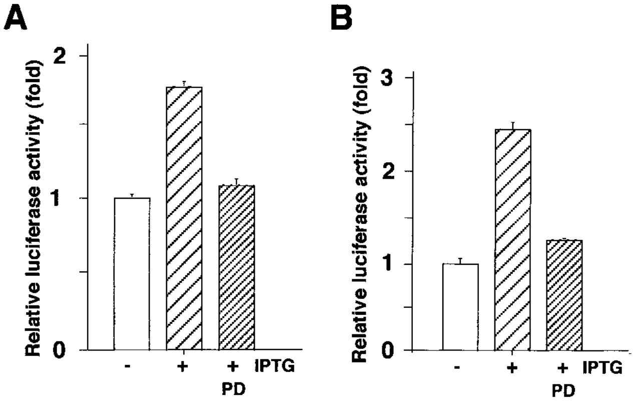

Fig. 6. The role of ERK in K-Ras-mediated induction of COX-2. *A,* inhibition of COX-2 transcription by PD 98059. A reporter construct containing the 5′-flanking region of the human *COX-2* gene (nucleotides −1432 to +59) was stably transfected into IEC-iK-Ras cells. Pooled cells were treated with vehicle (−), IPTG, or IPTG plus PD 98059. Firefly luciferase values were standardized to the controls and presented as mean ± SE of assays performed in quadruplicate. The results were similar in three separate experiments. *B,* inhibition of Ras-induced stabilization of COX-2 mRNA by PD 98059. A reporter construct containing the COX-2 3′ UTR (1.4 kb) was stably transfected into IEC-iK-Ras cells. Pooled cells were treated with vehicle, IPTG, or IPTG plus PD 98059. Firefly luciferase values were standardized to the controls and plotted as mean ± SE of quadruplicate assays. This experiment was repeated three times.

COX-2 mRNA by Ras (10). Consistent with these findings, expression of oncogenic K-Ras increased both the transcriptional activity of the *COX-2* promoter and the stability of COX-2 mRNA in IEC cells.

Our results provide evidence that Akt/PKB activity plays an important role in K-Ras-induced expression of COX-2. Treatment with LY 294002 partially blocks the induction of COX-2 by oncogenic K-Ras. Expressing a dominant negative mutant of Akt (Akt-K179M) significantly blocked the K-Ras-induced elevation of COX-2 expression, suggesting that Akt activity is required for the maximal induction of COX-2 by K-Ras. The results from transient transfection experiments clearly show that regulation of COX-2 expression by Akt/PKB occurs predominantly by modulation of the stability of COX-2 mRNA. Expression of Akt-K179M reduced the stability of COX-2 3′ UTR and blocked the Ras-induced stabilization of COX-2 3′ UTR, whereas expression of active Akt-myr greatly increased the stability of COX-2 3′ UTR. Further induction of K-Ras^{Val12} only exerted a limited effect on the stability of the COX-2 3′ UTR. These findings are strongly supported by the results obtained from transfection studies using a dominant negative PI3K construct. Inhibition of PI3K activity also blocked the K-Ras-induced stabilization of COX-2 3′ UTR but does not affect the transcription of *COX-2*, confirming the importance of the PI3K/Akt/PKB pathway for the regulation of COX-2 mRNA stability.

In summary, *COX-2* is a K-Ras targeted gene and is up-regulated by the induction of oncogenic K-Ras. Expression of mutated K-Ras activates the Rac1/MEKK1/JNK and Raf/MEK/ERK pathways that result in increased transcription of *COX-2*. Oncogenic K-Ras also activates the PI3K/Akt/PKB pathway, which cooperates with the MEK/ERK pathway and results in posttranscriptional stabilization of COX-2 mRNA (Fig. 8). Given the important roles of both COX-2 and Akt in carcinogenesis, our results suggest that COX-2 is regulated by PI3K/Akt/PKB and may contribute to the neoplastic potential of the PI3K/Akt/PKB pathway.

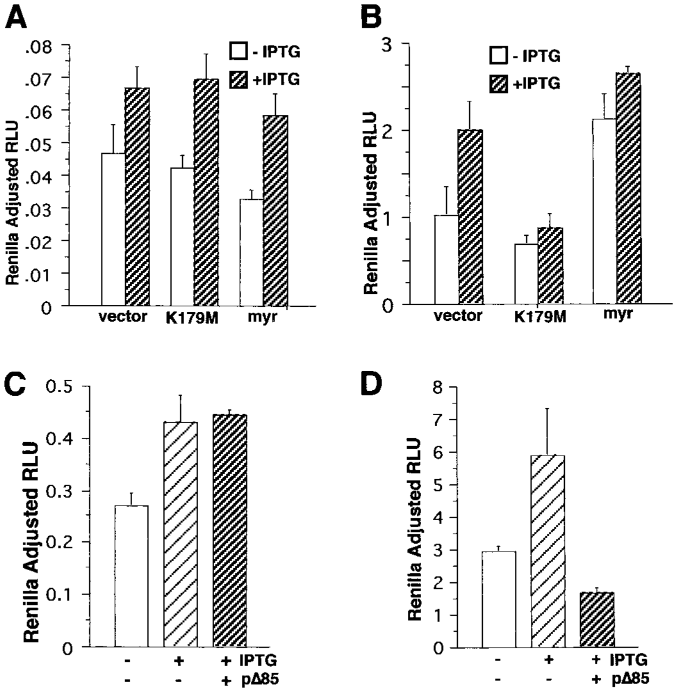

Fig. 7. The role of Akt/PKB and PI3K in the regulation of COX-2 by K-Ras. *A*, regulation of *COX-2* promoter activity by Akt. IEC-iK-Ras cells were cotransfected with phPES2(−1472/+59), pRL-CMV plasmid, and empty vector, dominant negative Akt construct (pCMV-Akt-K179M), or constitutively active Akt (pCMV-Akt-myr). After the cells were grown in the presence or absence of IPTG for 24 h, firefly luciferase values were measured and standardized to renilla values. The mean ± SE of assays performed in quadruplicate are plotted. The alteration of renilla luciferase activity resulted from the transfection of Akt-myr and Akt-K179M was <10%. All transient transfection experiments in this Figure were repeated at least three times. *B*, regulation of the stability of COX-2 mRNA by Akt. IEC-iK-Ras cells were cotransfected with pcDNA3/Luc+3′ UTR, pRL-CMV plasmids, and empty vector, pCMV-Akt-K179M, or pCMV-Akt-myr. After a 24 h incubation the cells were lysed. Firefly luciferase values were measured and standardized to renilla values. The mean ± SE of assays performed in quadruplicate are plotted. *C*, regulation of *COX*-2 promoter activity by PI3K. IEC-iK-Ras cells were cotransfected with phPES2(−1472/+59), pRL-CMV plasmid, and empty vector or dominant negative PI3K construct (pSG5-Δp85). After a 24 h incubation, firefly luciferase values were measured and standardized to renilla values. The mean ± SE of assays performed in quadruplicate are plotted. The alteration of renilla luciferase activity resulted from the transfection of pCMV-Δp85 was less than 10%. *D*, regulation of the stability of COX-2 3′UTR by PI3K. IEC-iK-Ras cells were cotransfected with pcDNA3/Luc+3′ UTR, pRL-CMV plasmid, and empty vector or dominant negative PI3K construct (pCMV-Δp85). After a 24 h incubation firefly luciferase values were measured and standardized to renilla values. The mean ± SE of assays performed in quadruplicate are plotted.

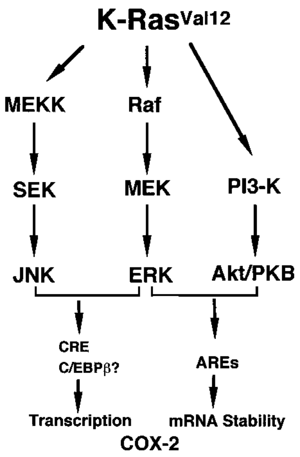

Fig. 8. Schematic diagram outlining the Ras-mediated regulation of COX-2 expression.

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