Elevated cyclooxygenase-2 (COX-2) expression and activity have been observed in several different transformed cell types that express mutated \textit{ras} genes. To investigate the mechanism of increased COX-2 expression following Ras-mediated transformation, \textit{Rat-1:iRas} cell line was transfected with an \textit{Ha-Ras}\textsuperscript{Val-12} cDNA expression vector that is under the transcriptional control of the lac operon and is inducible with isopropyl-1-thio-\textit{β}-D-galactopyranoside (IPTG). IPTG treatment caused parallel increases in the levels of Ha-Ras and COX-2 proteins in \textit{Rat-1:iRas} cells. The increased expression of COX-2 was accompanied by increased prostaglandin \textit{E}\textsubscript{2} production. Selective inhibition of COX-2 activity suppressed the production of prostaglandin \textit{E}\textsubscript{2} by >90% but did not alter the progress of the morphological transformation. The level of COX-2 mRNA was up-regulated by activated \textit{Ha-Ras}. Induction of \textit{Ras} increased the transcription of COX-2 by 44.3 ± 1.0% and increased the half-life of COX-2 mRNA by ~3.5-fold. A specific mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) inhibitor (PD 98059) caused a delay in both the activation of ERK1/2 and the induction of COX-2 in IPTG-induced \textit{Rat-1:iRas} cells. Inhibition of ERK activity by PD 98059 also suppressed the induction of COX-2 by epidermal growth factor in intestinal epithelial cells and significantly reduced the expression of COX-2 in \textit{Ha-Ras}-transformed rat intestinal epithelial cells. ERK activity appears to be required for induction of COX-2 by \textit{Ras}.

\textit{Ras} proteins are GDP/GTP-regulated switch molecules that relay signals from receptor tyrosine kinases to the nucleus via activation of a number of signal transduction cascades. \textit{Ras} activation stimulates both cell proliferation and differentiation (1–9). Receptor-mediated activation of \textit{Ras} leads to the sequential activation of Raf-1 serine/threonine kinase, mitogen-activated protein (MAP)\textsuperscript{1} kinase kinase (MEK1 and MEK2), and MAP kinases (4, 5). Activated p42 and p44 MAP kinases (also referred to as extracellular signal-regulated kinases (ERKs)) translocate into the nucleus, modulate the phosphorylation of transcription factors, and ultimately lead to the expression of genes that are crucial for cell growth and differentiation (6, 7). The stress-activated protein kinase (SAPKs)/c-Jun amino-terminal protein kinase (JNK) pathway is another kinase cascade distantly related to the MAPK pathway (8, 9). This pathway can be activated in a Ras-dependent or Ras-independent manner by extracellular stimuli (10, 11). MEKK1 (MEK kinase-1) phosphorylates SEK1 (SAPK/ERK kinase-1), a SAPK activator and in turn phosphorylates and activates SAPK (9).

\textit{Ras} mutations are found in a wide variety of human malignancies, with the highest incidences observed in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%) (12). Oncogenic mutations in \textit{Ras} result in constitutive activity of this small GTPase resulting in activation of downstream signaling proteins, including Raf and the downstream ERKs, as well as Raf-independent signaling proteins, including the Rho family proteins, RhoA, Rho B, and Rac1 among others, to modulate the expression of a specific subset of genes, and ultimately cause oncogenic transformation (13–18). Forced overexpression of oncogenic \textit{Ras} causes malignant transformation in multiple cell types including murine and rat fibroblasts (19, 20), rat intestinal epithelial cells (21, 22), and mammary epithelial cells (23).

Although much has been learned about \textit{Ras} signal transduction pathways, the specific target genes and proteins that contribute to the transformed phenotype are largely unknown (24, 25). Cyclooxygenase-2 (COX-2) is thought to be an important \textit{Ras} target gene (23, 26). Numerous studies have suggested that cyclooxygenase activity and/or prostaglandin synthesis may be pathogenic in numerous tumor types including colorectal carcinoma (27–29), breast cancer (23), and cancer of head and neck origin (30). Cyclooxygenase catalyzes the conversion of arachidonate to PGH\textsubscript{2}, the immediate precursor to prostaglandins, HETEs, and other eicosanoids. Two isoforms of cyclooxygenase, COX-1 and COX-2, have been identified (reviewed in Ref. 31). COX-1 is constitutively expressed in a variety of cells and tissues (32), whereas COX-2 is induced by cytokines, growth factors, and tumor promoters (31, 33). Up-regulation of COX-2 is a downstream effect of \textit{Ras}-mediated transformation in intestinal epithelial cells (RIE-1) (26), mammalian cells (34, 35), and prostate cells (36). COX-2 expression appears to be required for \textit{Ras}-mediated transformation in intestinal epithelial cells (RIE-1) (26), mammalian cells (34, 35), and prostate cells (36).

\begin{thebibliography}

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\bibitem{2} To whom correspondence should be addressed: Dept. of Surgery, CC-2306, Medical Center North, 1161 21st Ave. S, Vanderbilt University Medical Center, Nashville, Tennessee 37232.

\bibitem{3} The abbreviations used are: MAP, mitogen-activated protein; \textit{Rat-1:iRas}, \textit{Rat-1} fibroblast transfected with an inducible activated \textit{Ha-Ras}\textsuperscript{Val-12} cDNA; COX, cyclooxygenase; IPTG, isopropyl-1-thio-\textit{β}-D-galactopyranoside; MAPK, mitogen-activated protein kinase; SAPKs, stress-activated protein kinase; JNK, c-Jun amino-terminal protein kinase; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s Modified Eagle’s medium; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; PD 98059, (2-amino-3-methoxyflavone); HETE, hydroxyeicosatetraenoic acid; kβ, kilobase pair; PBS, fetal bovine serum; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; DRB, dichlorobenzimidazole riboside; PBS, phosphate-buffered saline; ARE, \textit{A} + U-rich elements; CRE, cyclic AMP response element; RIE, rat intestinal epithelial.

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mary epithelial cells (C57/MG) (23), and non-small cell lung cancer cells (34). Selective inhibition of COX-2 activity leads to programmed cell death in non-transformed intestinal cells (35) and similar effects along with decreased tumorigenicity for transformed intestinal epithelial cells that express high levels of COX-2 (26, 36, 37) implying that COX-2 may play an important role in the survival and growth of transformed intestinal epithelial cells.

In this study, we have attempted to investigate the mechanism for the increased expression of COX-2 during conditional Ras-mediated transformation of Rat-1 fibroblasts. Rat-1 fibroblasts were stably transfected with an inducible mutant Ha-RasVal12 cDNA expression vector. In this system the oncogene is under the transcriptional control of the Lac operon and can be strongly induced by treatment of the cells with isopropyl-1-thio-β-D-galactopyranoside (IPTG). This conditionally transformed cell line (Rat-1:iRas) exhibits a non-transformed phenotype under normal culture conditions and undergoes rapid transformation after the induction of mutated Ha-Ras by treatment with IPTG (24, 25). We found that COX-2 mRNA and protein expression and prostaglandin E2 production were greatly increased in parallel with the induction of activated Ha-Ras. Increased expression of COX-2 was the result of a modest increase in the rate of COX-2 gene transcription combined with stabilization of COX-2 mRNA. Finally, biochemical analysis indicates that activation of the MAPK pathway (ERK1/2) appears to be necessary for Ras or EGF-receptor mediated induction of COX-2 expression.

**Experimental Procedures**

**Cell Culture**—Rat-1:iRas cell line with an inducible activated Ha-RasVal12 cDNA was a generous gift from Dr. I. Hiroki of Tokyo University of Technology (24, 25). The Ha-RasVal12 cDNA is under the transcriptional control of the Lac operon in a eukaryotic expression system (Stratagene, La Jolla, CA). Rat-1:iRas cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 400 μg/ml G418 (Life Technologies, Inc.), and 150 μg/ml hygromycin B (Calbiochem) as described previously (25). IPTG (isopropyl-1-thio-β-D-galactopyranoside, Life Technologies, Inc.) at a concentration of 1 mm was used to induce the expression of mutated Ha-Ras. A nontransformed rat intestinal epithelial (RIE-1) cell line was maintained in DMEM with 10% FBS. The mutated Ha-Ras-transformed RIE-1 cells were stably transfected with Ha-RasVal12 cDNA under the transcriptional control of RSV promoter and maintained in DMEM with 10% FBS and 500 μg/ml of G418.

**RNA Extraction and Northern Blot Analysis**—Total cellular RNA was extracted according to Chirgwin et al. (38). RNA samples (20 μg per lane) were separated on formaldehyde-agarose gels and blotted onto nitrocellulose membranes. The blots were hybridized with cDNA probes labeled with [α-32P]dCTP by random primer extension (Stratagene, La Jolla, CA). After hybridization and washes, the blots were subjected to autoradiography. 18 S rRNA signals were used as controls to determine integrity of RNA and equality of the loading. For determination of mRNA stability, Rat-1:iRas cells were treated with IPTG or vehicle for 8 h and then the transcription was stopped by addition of 40 μM DRB (dichlorobenzimidazole riboside, Sigma). The RNA samples were isolated every 10–15 min following the DRB treatment and analyzed for mRNA levels by Northern blotting.

**Immunoblot Analysis**—Immunoblot analysis was performed as described previously (39). Briefly, the cells were lysed for 30 min in radio-immunoprecipitation assay buffer (RIPA, 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μM sodium orthovanadate), and then clarified cell lysates were denatured and fractionated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membrane. The filters were then probed with the indicated antibodies, detected by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech), and exposed to X-AR5 film (Kodak). Quantitation was by densitometry. The anti-COX-2 antibodies and anti-CDK4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pan Ras antibody was purchased from Calbiochem. Anti-active MAPK antibody was purchased from Promega (Madison, WI).

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**Immunofluorescence**—Rat-1:iRas cells were grown in 35-mm tissue culture plates and fixed in methanol/acetic acid at −20 °C for 10 min. Fixed cells were incubated with 10% normal donkey serum for 1 h and then with anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI) for 2 h at room temperature. After washing the cells three times with PBS of PBS containing 1% Tween 20, the cells were incubated with Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for an additional hour. The cells were washed with PBS, mounted, and observed under fluorescent microscopy with appropriate filters.

**Quantitation of Eicosanoids**—Subconfluent cell cultures were established; the cells were treated with 1 μM SC-58125, a selective COX-2 inhibitor, [1-(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)isoindole (Searle), or vehicle for 24 h. Serum-free DMEM with 15 μT arachidonic acid (Cayman Chemical, Ann Arbor, MI) was replaced 1 h prior to collecting the conditioned medium. The PGE2 formation in medium was quantitated by utilizing stable isotope dilution techniques employing gas chromatography negative ion chemical ionization mass spectrometry. The results are expressed as nanograms of prostaglandin E2 per ml of medium.

**Transfection of Reporter Constructs**—Reporter constructs were generated from a plasmid containing 2.7 kb of rat COX-2 5′-flanking sequence (obtained as a generous gift from JoAnne Richards). Deletion constructs of the COX-2 promoter were generated by polymerase chain reaction using a blend of Pfu/Taq polymerase (Takara, Shiga, Japan). KpnI and XhoI restriction sites were engineered onto the tails of the primers. After digestion the polymerase chain reaction products were ligated into pGL3 Basic luciferase plasmid (Promega, Madison WI). Sequence validity was ascertained via direct sequencing. A total of five constructs were generated as follows: −2700 Cox-2/luc, −619 Cox-2/luc, −446 Cox-2/luc, −289 Cox-2/luc, −147 Cox-2/luc each containing 2700, 619, 446, 289, and 147 base pairs upstream of the transcriptional activation site of the rat cox-2 gene. For transient transfections, cells were plated in 24-well plates 24 h prior to transfection and then co-transfected with 0.5 μg of one of the COX-2 promoter firefly luciferase plasmid constructs, pGL3 Basic plasmid, or pGL3 control plasmid, and 0.25 μg of pRL-TK plasmid, containing a herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene (Promega, Madison WI), using the Lipofect procedure (Life Technologies, Inc.) as described in the manufacturer’s protocol. Transfected cells were cultured for 12 h in medium containing 10% FBS to allow recovery. The cells were switched to serum-free medium for 4 h, and then one plate was stimulated with 5 mM IPTG 24 h later the cells were washed twice with 3 ml of Ca2+- and Mg2+-free PBS and lysed with passive lysis buffer (Promega, Madison WI). 20 μl of lysate was used for both the firefly and renilla luciferase readings. Firefly and renilla luciferase activities were measured using a Dual-Luciferase Reporter assay system (Promega, Madison WI) and a model TD/20/20 Luminometer (Turner Design). Firefly luciferase values were standardized to renilla values.

**Inhibition of MAPK Pathway**—PD 98059 (Calbiochem), a specific inhibitor of mitogen-activated protein kinase kinase, was used for blocking the MAPK pathway activated by mutated Ha-Ras in Rat-1: iRas cells. PD 98059 at 75 μM concentration in MeSO was added to the culture media 1 h prior to induction of Ha-Ras by IPTG treatment. The same volume of MeSO was added to the control cells. The media containing IPTG and PD 98059 were replaced daily. The morphology was observed on a daily basis. Protein lysates were collected for detection of COX-2. A similar protocol was followed for the epidermal growth factor (EGF, Sigma)-treated RIE-1 cells.

**MAP Kinase Activity Assay**—p42/p44 MAP kinase activity was measured by determining the transfer of the phosphate group of adenosine 5′-triphosphate to a peptide that is highly specific substrate for the p42/44 MAP kinase (Biotek System, Amersham Pharmacia Biotech).
RESULTS

Rapid Induction of COX-2 Protein by Activated Ha-Ras—We previously reported that COX-2 expression is increased more than 12-fold in an Ha-Ras-transfected rat intestinal epithelial cell line (26). To determine whether induction of COX-2 is an important early event in activated Ras-induced transformation, we used Rat-1 cells stably transfected with an inducible Ha-Ras expression construct (Rat-1:iRas). Previous studies have shown that addition of IPTG into the culture medium rapidly induces expression of mutated Ha-Ras and subsequent morphological transformation in these cells (24, 25). We used these cells to determine the temporal expression of COX-2 in the context of activated Ha-Ras. As shown in Fig. 1A, COX-2 was expressed at very low levels in the parental Rat-1 fibroblasts. IPTG treatment for 24 h slightly reduced the expression of COX-2 in parental Rat-1 cells. In Rat-1:iRas cells, addition of IPTG into the culture medium induced activated Ha-Ras protein by 2 h. Thereafter, the level of Ras protein was continuously elevated for the duration of IPTG treatment (Fig. 1B). The effect of Ras induction on the expression of COX-2 is demonstrated in Fig. 1, A and C. The elevation of COX-2 protein was detected 4 h after addition of IPTG. The induction of COX-2 temporally coincided with the induction of mutated Ha-Ras protein. The expression of cyclin-dependent kinase 4 (cdk4) was unchanged during induction of Ha-Ras and is shown as a loading control (Fig. 1, B and C). Immunofluorescence staining demonstrates that the COX-2 protein predominantly was located in perinuclear cytoplasm, and COX-2 immunoreactivity was increased by 8 h after addition of IPTG. Within 24 h after removal of IPTG, COX-2 immunoreactivity returned to the uninduced basal level (Fig. 1D).

Inhibition of COX-2 Activity—Analysis of eicosanoid production revealed that PGE$_2$ was the predominant prostanoid produced in both transformed and non-transformed Rat-1:iRas cells. Upon induction of mutated Ras with IPTG, PGE$_2$ levels increased 1.4-fold by 4 h, 3.0-fold at 8 h, and 3.1-fold at 24 h (Fig. 2A). A selective COX-2 inhibitor, SC-58125 (42, 43), at 1 μM inhibited the conversion of prostaglandin E$_2$ from arachidonic acid by >90%, confirming that the elevated prostanoid production was caused by the increased COX-2 activity.

To determine the role of COX-2 activity in Ras-mediated transformation, Rat-1:iRas cells were treated with IPTG in the presence or absence of SC-58125 (1, 10, and 25 μM). The morphological alteration was recorded at 24 and 48 h. As demonstrated in Fig. 2B, the morphological transformation of the Rat-1:iRas cells was observed within 24 h of IPTG treatment. Celi-cell contact inhibition was lost, and the cells acquired a spindly appearance and grew in overlapping clusters. After 48 h of IPTG treatment the cells formed large foci. Addition of SC-58125 at 25 μM neither delayed the progress nor reduced the degree of morphological transformation. Increased prostanoid production by COX-2 did not appear to be a necessary factor for Ha-Ras-mediated morphological transformation in Rat-1 fibroblasts.

Mechanism of Induction of COX-2 by Activated Ha-Ras—We next investigated the mechanism by which Ras activation increased levels of COX-2 protein in Rat-1:Ras cells. Northern analyses for COX-1 and COX-2 messages are demonstrated in Fig. 3A. The expression of COX-2 mRNA was barely detectable in untreated Rat-1:Ras cells. The induction of Ras by IPTG coincided with an increase in the level of COX-2 mRNA by 2.3-fold at 4 h and 5.2-fold at both 8 and 24 h. In contrast, COX-1 mRNA level remained low and was not affected by Ras induction. To determine whether induction of oncogenic Ha-Ras resulted in stabilization of COX-2 message RNA, 5,6-dichlorobenzimidazole riboside (DBR at 40 μM) was used to in-
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Fig. 2. Prostaglandin synthesis and transformation. A, PGE$_2$ production in Rat-1:iRas cells. Cells were treated with IPTG and 1 $\mu$m SC-58125 (open squares) or Me$_2$SO (open circles) for the indicated times. Medium was replaced with serum-free DMEM containing 15 $\mu$m arachidonic acid 1 h prior to collection. The PGE$_2$ formation in the medium was quantified by utilizing stable isotope dilution techniques employing gas chromatography-negative ion chemical ionization mass spectrometry. The results are expressed as nanograms of prostaglandin E$_2$ per ml of medium. B, morphological transformation of Rat-1:iRas cells. Rat-1:iRas cells were grown on 60-mm tissue culture dishes. The cells were treated with 5 mM IPTG for 24 (B) and 48 h (C) or 5 mM IPTG plus 25 $\mu$m of SC-58125 for 24 h (D) and 48 h (E). SC58125 and IPTG were readed every 24 h. The pictures were taken by using an inverted microscope (original magnification, $\times$ 100). A, shows the morphology of untreated Rat-1:iRas cells.

Fig. 3. Northern analyses of COX-1, COX-2, and Jun B mRNAs in Rat-1:iRas cells. A, cells were treated with 5 mM IPTG for the indicated hours. Total RNA was isolated for detection of COX-1, COX-2, and Jun B mRNA expression. B, degradation of COX-2 and Jun B mRNA. Rat-1:iRas cells were treated with IPTG (+) or vehicle (−) for 8 h. Then transcription was stopped by addition of 40 $\mu$m DRB. The RNA samples were isolated at the indicated time points following DRB treatment for detection of COX-2 and Jun B mRNA levels. Since IPTG-treated Rat-1:iRas cells expressed higher levels of COX-2 mRNA as compared with uninduced Rat-1:iRas cells prior to the DRB treatment, the autoradiograms of control RNA blots for COX-2 were exposed 6 times longer than the autoradiograms of IPTG-treated RNA blots. As demonstrated in Fig. 3B, the Ha-Ras induction caused a decrease in the rate of COX-2 mRNA degradation as compared with the degradation rate in untreated Rat-1:iRas cells. The level of COX-2 mRNA was decreased by 50% after 40 min of DRB treatment of Rat-1:iRas cells in the presence of Ras induction. The $t_1/2$ of COX-2 mRNA was estimated to be 6 min in the absence of Ras induction. Induction of activated Ha-Ras oncogene prolonged the half-life of COX-2 mRNA to > 20 min (−3.5-fold). An immediate early gene jun B was induced by Ha-Ras at mRNA levels (Fig. 3A). 50% of Jun B mRNA was degraded by 20 min, and Ha-Ras induction increased the $t_1/2$ of Jun B mRNA to slightly over 20 min (Fig. 3B). Increased COX-2 mRNA stabilization may not be the sole mechanism whereby COX-2 message is increased. In order to determine whether activated Ha-Ras increased the transcription of COX-2, nuclear run-on transcription assays were conducted. Rat-1:iRas cells were treated with IPTG or vehicle for 6 or 24 h, and radioactive labeled nascent transcripts were analyzed by hybridization to immobilized DNAs. cox-2 gene transcription at 6 and 24 h after induction of Ha-Ras was increased by 44.3 ± 10.1% (mean ± S.E., $n = 4$, $p < 0.005$) by activated Ha-Ras, whereas the transcription of cox-1 was not altered by activation of the Ras signaling pathway, as determined by four independent experiments. Transcription of the jun B gene was increased 2.9-fold at 6 and 24 h after induction of onecogenic Ha-Ras. Fig. 4A represents a representative autoradiogram of the experiment.

To evaluate further the role of transcriptional induction in the COX-2 mRNA increase, COX-2 promoter/luciferase constructs were transfected into Rat-1:iRas cells. Luciferase activity was determined after either no stimulation or 24 h of IPTG-induced Ha-Ras expression. A slight transcriptional effect was observed, with a 2.7-kb COX-2 luciferase construct showing a 1.3-fold induction and a -619 COX-2 construct showing a maximal induction of 2.5-fold (Fig. 4B). These results suggest that activation of Ha-Ras oncogene induces an increase in COX-2 in Rat-1:iRas cells and is caused by a combination of mRNA stabilization and a modest transcriptional induction.

The Role of MAPK Pathway—Transformation by activated
Ras involves a phosphorylation cascade that leads to activation of multiple signaling pathways including mitogen-activated protein kinase (MEK/ERK) pathway. PD 98059, a specific inhibitor of mitogen-activated protein kinase kinase (MEK), has been shown to inhibit the activation of MEK both in vitro and in vivo (44, 45). It was of interest to study the effect of PD 98059 on the transformation of Rat-1:iRas cells and associated induction of cyclooxygenase-2. The morphological transformation of the Rat-1:iRas cells was observed within 24 h of IPTG treatment. Addition of PD 98059 at 75 μM prevented the cells from undergoing morphological transformation by 24 h but by 48–72 h the cells appeared transformed despite the continuous presence of PD 98059 (Fig. 5A).

Interestingly, PD 98059 treatment also delayed the induction of COX-2 at both mRNA and protein levels. As shown in Fig. 5B, IPTG treatment increased the level of COX-2 mRNA at 8, 24, and 48 h. The addition of PD 98059 prevented the induction of COX-2 mRNA at 8 and 24 h, but by 48 h after IPTG exposure in the presence of PD 98059, COX-2 mRNA levels reached 60% of the maximal levels observed in the absence of PD 98059. This delayed COX-2 expression coincided with delayed morphological transformation of the cells. Nuclear run-on assays demonstrated that PD 98059 (75 μM) blocked the Ha-Ras induced increase in COX-2 transcription when evaluated at 24 h following addition of IPTG (Fig. 5C). PD 98059 treatment also prevented the Ras-induced stabilization of COX-2 mRNA (Fig. 5D). Treatment of the IPTG-induced Rat-1:iRas cells with PD 98059 resulted in a less stable COX-2 mRNA (t1/2 <10 min) similar to that in Rat-1:iRas cells in which Ha-Ras has not been induced. Therefore, pharmacologic inhibition of the MEK/ERK pathway delayed both transcriptional activation and stabilization of COX-2 mRNA in Rat-1:iRas cells.

Next, immunoblot analyses were performed to determine whether ERK activation correlated with the expression of COX-2. As demonstrated in Fig. 6A, the lysates from uninduced Rat-1:iRas cells contained very low levels of activated 42- and 44-kDa ERK1/2, and PD 98059 treatment, as expected, further reduced the levels of activated ERK1/2. IPTG treatment significantly increased the levels of activated ERK1/2 from 8 through 72 h. The addition of 75 μM PD 98059 completely blocked the activation of ERK1/2 by MEK for at least 24 h. Despite the continued presence of PD 98059, the Ras-activated ERK levels began to recover from the inhibitory effect of PD 98059 by 48 h. The expression of Cox-2 appeared to increase in parallel with levels of active ERK1/2. PD 98059 inhibited the induction of COX-2 protein at 8 and 24 h following IPTG treatment; however, increased levels of COX-2 protein were observed by 48 and 72 h following Ras induction even in the presence of PD 98059 treatment. These increases in COX-2 levels correlated with the increases in activated ERK1/2 (Fig. 6A). In order to confirm these findings, MAPK activity was evaluated. Activation of Ha-Ras increased ERK1/2 kinase activity by 6–10-fold from 8 to 72 h following addition of IPTG. Addition of PD 98059 completely inhibited the ERK activity at 8 and 24 h; however, partial recovery of ERK activity occurred by 48 and 72 h with respective activity levels of 44.7 and 55.6% of the levels achieved by IPTG exposure in the absence of PD 98059 (Fig. 6B). The recovery of ERK1/2 activities from PD 98059 inhibitory effect coincided with the induction of COX-2 and morphological transformation in Rat-1:iRas cells. These results suggest that COX-2 induction is temporally related to the process of transformation but do not establish a causal relationship between COX-2 and transformed phenotype. We further examined this question by determining whether the COX-2 inhibition was able to prevent the morphological transformation of the Rat-1:iRas cells after IPTG treatment. We found that addition of COX-2 inhibitor SC-58125 at 10 μM did not delay or inhibit Ras-mediated morphological transformation in Rat-1:iRas cells.

**Fig. 4. Transcription of cox-2 gene in Rat-1:iRas cells treated with or without IPTG.** A, determination of COX-2 transcriptional levels by nuclear run-on assay. Rat-1:iRas cells were stimulated with vehicle or 5 mM IPTG for 6 and 24 h; nuclei were isolated, and in vitro run-on transcription was carried out by using 1 x 10^7 nuclei and 200 μCi of [α-32P]UTP/assay at 30 °C for 45 min. Labeled transcripts were purified by trichloroacetic acid precipitation. A total 1 x 10^7 cpm elongated nascent RNA per assay was hybridized for 48 h at 65 °C to filter-immobilized plasmid DNA. B, transient transfection assay. Reporter constructs containing different length of rat Cox-2 5'-flanking sequence were generated as described under “Experimental Procedures.” For transient transfection, cells were plated in 24-well plates 24 h prior to transfection and then co-transfected with 0.5 μg of one of the promoter firefly luciferase plasmid constructs, pGL3 basic vector or pGL3 control plasmid, and 0.25 μg of pRL-TK plasmid, containing a herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene, using the Lipofectin procedure. The cells were switched to serum-free medium for 48 h, and then one plate was stimulated with 5 mM IPTG. 24 h later the cells were washed twice with PBS and lysed with passive lysis buffer. 20 μl of lysate was used for both the firefly and renilla luciferase readings. Firefly luciferase values were standardized to renilla values. Plotted is the mean ± S.E. of assays performed in triplicate.

**Fig. 6B.** COX2 and ERK in Intestinal Epithelial Cells—Colorectal cancers often bear Ras mutations and have elevated expression of cyclooxygenase-2. In order to determine whether the above observations in fibroblasts were applicable to intestinal epithelial cells, further experiments were performed to determine whether activation of ERK1/2 is necessary for induction of COX-2 in intestinal epithelial cells. Growth factors known as ERK stimulators were used to activate the MEK/ERK pathway in RIE cells. Both EGF and TGF-α have been shown to stimulate the proliferation of rat intestinal epithelial (RIE-1) cells by
activating the MEK/ERK pathway and to a lesser degree the SAPK/JNK pathway (8). Both EGF and TGF-α can also induce COX-2 expression in intestinal epithelial cells (30, 46). Active 42- and 44-kDa ERK1/2 bands were abundantly detected by immunoblot analysis at 1, 3, and 6 h following addition of EGF (100 ng/ml) to the culture media (Fig. 7A). Associated with the increase in ERK1/2 activity, elevated expression of COX-2 protein was observed at 1, 3, and 6 h following EGF treatment. Addition of PD 98509 abrogated both increases in active ERK1/2 and induction of COX-2 after EGF stimulation. Similar results were obtained in the RIE-1 cells treated with TGF-α (data not shown).

It has been previously shown that COX-2 is expressed at high levels in RIE cells that are permanently transfected by constitutively expressed Hae-Ras (26). To determine whether the overexpression of COX-2 in Ras-transformed RIE cells depends upon MEK/ERK activation, Hae-RasVal-12-transfected RIE cells were examined. As shown in Fig. 7B, RIE- Hae-RasVal-12 cells abundantly express COX-2 protein. Addition of PD 98509 reduced the levels of COX-2 by 41.9% at 6 h and 67.8% at 24 h and decreased the amount of active ERK1/2 by >90%.

**DISCUSSION**

Identifying the target genes of Ras signaling and exploring their regulation by activated Ras is a key step in understanding the mechanism(s) whereby Ras gene mutations contribute to malignant transformation. In this study, using inducible activated Hae-RasVal-12-transfected Rat-1 cells, we found that activation of Hae-Ras results in rapid induction of COX-2 and prostaglandin E2 production. In addition, studies in non-small cell lung cancer cells have demonstrated that activated Ras increased expression of cytosolic phospholipase A2, an enzyme...
that releases arachidonic acid from cellular phospholipid stores, thus making it available for the cyclooxygenase enzymes. These two observations suggest that increased prostanoid production may be an important phenotypic consequence of Ras activation (34).

Although the expression of cyclooxygenase-2 is often associated with neoplastic transformation of epithelial cells, the precise role of COX-2 in transformation is not clear. We have previously reported that increased COX-2 expression is not sufficient for neoplastic transformation of rat intestinal epithelial cells (35), and selective inhibition of COX-2 activity did not reverse the transformed phenotype of Ha-Ras-transfected RIE cells (RIE-Ras) (26) or COX-2 expressing human colon cancer cells (HCA-7) (36). In this study, we found that selective inhibition of COX-2 activity by treatment of SC-58125 reduced the production of PGE2 by >90% but did not alter the progress of morphological transformation. These results suggest that induction of COX-2 is an early consequence of Ras activation in Rat-1 fibroblasts but is not required for morphological alterations that are indicative of the transformed phenotype. What role might induction of COX-2 play in tumor progression? In previous studies, forced expression of COX-2 in non-transformed intestinal epithelial cells (RIE-1) resulted in resistance to apoptosis (35). Selective inhibition of COX-2 activity suppressed the growth of RIE-Ras (26) and HCA-7 cells (36) primarily via the induction of apoptosis. Addition of prostaglandin E2, the predominant eicosanoid product of COX-2 in RIA-7 cells, significantly increased the clonogenicity of HCA-7 cells and abrogated the induction of apoptosis caused by treatment with a COX-2 antagonist (29). These findings suggest that increases in COX-2 and prostaglandins that occur during cell transformation may enhance cell survival and provide a selective advantage for the transformed cells that overexpress COX-2. Interestingly, the induction of COX-2 by Ha-Ras was not sufficient to prevent the Rat-1:Ras cells undergoing apoptosis beginning 72 h after IPTG treatment. Further studies will be required to determine whether COX-2 expression and prostaglandins provide selective protection for epithelial cells as compared with fibroblasts.

Previously, it was reported that COX-2 expression was regulated at the transcriptional level by activated Ha-Ras in mammary cells (25, 30) and by oncogene v-src (47) and growth factors (48) in NIH 3T3 cells. Both human and murine COX-2 promoters contain a consensus cyclic AMP response element (CRE). The CRE element in the murine COX-2 promoter is essential for optimal COX-2 gene expression in response to v-src and platelet-derived growth factor or serum (47, 48). The rat COX-2 promoter does not contain this CRE sequence implying that different regulatory pathways may be utilized for induction of COX-2 between murine and rat cells. Analysis of nuclear run-on experiments revealed that the basal transcription rate of COX-2 in Rat-1 fibroblasts was relatively high and was increased only modestly after Ras induction. In addition to modest transcriptional activation of COX-2, we also observed post-transcriptional stabilization of COX-2 mRNA after Ras induction. In non-transformed Rat-1:iRas cells, the COX-2 mRNA was very unstable and was quickly degraded, thus steady-state levels of COX-2 mRNA and protein were barely detectable. There was a -3.5-fold reduction in the COX-2 mRNA decay rate in Ras-transformed Rat-1 cells as compared with non-transformed Rat-1:iRas cells. These results suggest that activation of the Ras signal transduction pathway either induces factor(s) that stabilize or inhibits factors that destabilize COX-2 mRNA. Recent studies suggest that under selective conditions post-transcriptional regulation of mRNA stability may significantly contribute to the regulation of COX-2 mRNA levels. Interleukin-1a induced rapid but transient activation of COX-2 transcription and also prolonged the half-life of the COX-2 mRNA (49). Post-transcriptional regulation of cytokine-induced cyclooxygenase-2 transcript isoforms by dexamethasone has also been reported (50).

In the present study, we observed no change in the levels of COX-1 mRNA after Ha-Ras activation in Rat-1:iRas cells. Although COX-1 and COX-2 are very similar at the amino acid level, and even have comparable enzymatic activities, their expression pattern is markedly different. COX-1, often referred to as the “constitutive” cyclooxygenase, is present in most tissues. In contrast, COX-2 is normally absent but can be rapidly induced by a wide range of stimuli. The 3′-untranslated region of the COX-2 transcript differs from COX-1 and has two polyadenylation signals, which potentially explain different mRNA sizes observed by Northern analysis (2.8–4.5 kb). This region is extremely AT-rich, and contains 9 copies of the Shaw-Kamens sequence (ATTTTA) otherwise known as A+U-rich elements (AREs) (51, 52). This motif is present in many immediate early genes and is thought to be involved in regulating the rate of mRNA degradation. For example, if AREs are added to a normally stable transcript, such as β-globin, the transcript is rapidly degraded (53). It is of interest to note that these ARE sequences are conserved between murine, rat, and human COX-2 3′-untranslated regions, suggesting an important role for their presence.

The mechanism by which mutated Ras induces transformation is complex and incompletely understood. Different Ras effectors may be required for transformation of different cell types. Activation of the Raf-1/MAP kinase cascade alone is not sufficient for Ras transformation of rat intestinal epithelial cells but appears to be sufficient for the transformation of rodent fibroblasts (54). A selective MEK inhibitor PD 98059 reverses the phenotype of Ras-transformed BALB 3T3 mouse fibroblasts and rat kidney cells (44). Activated Ras promotes the translocation of Raf-1 to the plasma membrane (55, 56),

![FIG. 7. ERK1/2 activation and expression of COX-2 in epithelial cells. A, induction of active ERK1/2 and COX-2 by EGF in RIE-1 cells. Subconfluent RIE-1 cells were treated by 100 ng/ml EGF or EGF and PD 98059. Cell lysates were collected at the time points indicated and subjected to Western analyses for active ERK1/2 and COX-2. B, effect of PD 98059 on the expression of COX-2 in Ha-Ras-transformed RIE-1 cells. RIE-1 cells stably transfected with mutated Ha-RasVal12 DNA were treated by addition of 75 μM PD 98059. The levels of active ERK1/2 and COX-2 were determined by Western blotting at the indicated times.](image)
additional factors complete the activation of Raf-1 kinase activity (57), and then Raf-1 in turn phosphorylates MEK1 and -2. Activated Ras also activates Rho family proteins (RhoA, Rho B, and Rac1), and the Rho proteins phosphorylate and activate PAKs and MEKK1–3. MEKK1–3 are able to activate both JNK/SAPK and ERK (13, 15). We observed that morphological transformation of Rat-1:Ras cells was accompanied by an increase in ERK activity indicating the importance of the Ras-Raf-MEK-ERK pathway in oncogenic Ha-Ras-induced transformation of Rat-1 cells. Treatment with the MEK inhibitor, PD 98059, delayed both the induction of active ERK and the morphological transformation of IPTG induced Rat-1:Ras cells, but after 24 h the ERK activity recovered and the cells acquired the transformed phenotype despite repeatedly adding PD 98059. The PD 98059 compound does not inhibit phosphorylated MEK but inhibits both activation and phosphorylation of MEK by Raf kinase and MEKK. Whereas PD 98059 potently inhibits the activation of MEK1, it has much less inhibitory activity for MEK2 (45). The incomplete transient inhibition of ERK activation by PD98059 may be caused either by incomplete suppression of Raf and MEK activities or by other Raf-independent Ras signaling pathways (58) that may not be suppressed by PD 98059 treatment.

Previously Xie and Herschman (47, 48) reported that induc- tion of COX-2 by the oncogene v-src (47) and platelet-derived growth factor or serum (48) in NIH 3T3 cells requires activation of both Ras/Rac1/MEKK1/JNK and Ras/Raf1/ERK signal transduction pathways. Our studies suggest that both the transcriptional and post-transcriptional regulation of COX-2 expression by activated Ha-Ras is dependent on the MAPK. Complete inhibition of MEK/ERK activity by PD 98059 between 8 and 24 following treatment abolished both the increased transcriptional activity and the stabilization of COX-2 mRNA. When the activation of ERK escaped from PD 98059 inhibitory effect, the levels of COX-2 mRNA and protein increased. Our results do not exclude the possibility that JNK or other signaling pathways are also required for full expression of COX-2 in Ras-transformed Rat-1 cells (47, 48). In addition, we also found that MAPK activity is necessary for the high level expression of COX-2 in intestinal epithelial cells that have been transfected with activated Ha-Ras or in EGF-stimulated non-transformed rat intestinal epithelial cells. Our observations suggest that induction of MAPK activity is essential for induction of COX-2 expression that occurs after activation of the Ras pathway, whether by induction of JNK or other signal-