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ARTICLES

Differential Regulation of Cyclooxygenase 2 Expression by Small GTPases Ras, Rac1, and RhoA

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Abstract Cyclooxygenase 2 (COX-2) is an immediate early gene induced by a variety of stimuli and its expression is stimulated by individual activation of Ras or Rho GTPases. Here we investigate the role of coordinate activation of Ras and Rho GTPases in the induction of COX-2. Individual expression of constitutively active Ras, RhoA, or Rac1 was capable of stimulating COX-2 expression in NIH3T3 cells, but co-expression of constitutively active RhoA with either constitutively active Ras or Rac1 was required for full stimulation of COX-2 expression. Serum growth factors differentially activated Ras, RhoA, and Rac1, which correlated with the activation of Raf-1, ERK, and c-Jun as well as with induction of COX-2. Inhibition of Ras significantly blocked the activation of Raf-1, ERK, and c-Jun and the stimulation of COX-2 expression in response to serum. In contrast, inhibition of Rho family GTPases partially blocked serum induction of ERK activation but had little effects on COX-2 expression. Both inhibitors of MEK (PD098059) and JNK (SP600125) inhibited serum induction of COX-2. PD98059 only inhibited constitutively active Ras-induced COX-2 expression, while SP600125 significantly inhibited both constitutively active Ras- and RhoA-induced COX-2 expression. Together, our data suggest that constitutively active oncogenic Ras and Rho coordinately stimulate COX-2 expression whereas transient activation of Ras but not RhoA or Rac1 mediates the induction of COX-2 in response to serum. Furthermore, ERK and JNK activation are both required for serum- and oncogenic Ras-mediated COX-2 expression whereas only JNK activation is required for oncogenic RhoA-mediated stimulation of COX-2 expression. J. Cell. Biochem. 96: 314–329, 2005.

Key words: cyclooxygenase 2; Ras and Rho GTPases; ERK; JNK

Cyclooxygenase (COX) also known as prostaglandin (PG) H synthase catalyzes the rate-limiting step in the conversion of arachidonic acid to PGs and related eicosanoids. PGs are arachidonic acid metabolites that regulate cell division, inflammatory responses, bone development, wound healing, hemostasis, reproductive function, glomerular filtration, and renal homeostasis [Dubois et al., 1998]. Alterations in PG production have been linked to cardiovascular disease, chronic and acute inflammation, atherosclerosis, and colon cancer [Dubois et al., 1998]. Two isoforms of COX have been identified and cloned in eukaryotic cells. COX-1 is constitutively expressed in most cell types and is involved in the maintenance of physiological functions. In contrast, COX-2 is an immediate early gene and its expression is induced by serum, platelet derived growth factor (PDGF), proinflammatory cytokines, tumor promoters, and oncogenes (such as v-src) [Dubois et al.,

Abbreviations used: COX, cyclooxygenase; PGs, prostaglandins; PDGF, platelet derived growth factor; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; JNK, c-JUN N-terminal protein kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TBS, Tris-based buffer saline; BSA, bovine serum albumin; BCA, bicinchoninic acid; HRP, horseradish peroxidase; GST, glutathione S-transferase; RBD, Ras binding domain or Rho binding domain; PAK, p21 activated kinase; PBD, p21 binding domain; ROCK, Rho kinase.

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1998]. Furthermore, overexpression of COX-2 has been observed in colorectal, gastric, pancreatic, lung, breast, prostate, and skin cancers [Eberhart et al., 1994; Ristimaki et al., 1997; Chan et al., 1999; Yoshimura et al., 2000; Half et al., 2002]. Several reports using animal models have shown that treatment with selective COX-2 inhibitors results in a significant decrease in tumor growth [DuBois, 2003]. These observations strongly suggest that COX-2 overexpression is an important factor in tumorigenesis.

Although the molecular mechanisms mediating the up-regulation of COX-2 expression are not fully understood, previous studies have shown that induction of COX-2 correlates with activation of Ras or Rho signaling pathways [Sheng et al., 1998, 2001; Slice et al., 2000, 2005; Hahn et al., 2002; Aznar Benitah et al., 2003]. The Ras superfamily of small GTPases are guanine nucleotide-binding proteins and play key roles in regulating a variety of essential biochemical pathways in eukaryotic cells. Among the 100 or so small GTPases, which have been identified in mammalian cells, the Ras and the Rho families are of special interest because they transduce the signals from external stimuli to intracellular signaling pathways to regulate diverse cellular functions [Bar-Sagi and Hall, 2000]. The Ras GTPase family plays an important role in coupling the signals from receptor tyrosine kinases to the nucleus to stimulate proliferation and differentiation. Ras mediates its effect on cell growth in part by activation of a cascade of kinases: Raf, MAPK/ERK kinases, and ERK. Mutations in Ras are found in a wide variety of human malignancies, such as adenocarcinomas of the pancreas, the colon, and the lung [Vojtek and Der, 1998]. Oncogenic mutations in Ras inhibit GTPase activity and lock Ras in its active form, resulting in a growth-factor-independent, constitutively active signal that leads to uncontrolled growth and malignant transformation [Vojtek and Der, 1998]. The Rho family includes RhoA, Rac1, and Cdc42 and is involved in regulation of the actin cytoskeleton organization, cell growth, vesicular cell trafficking, and transcriptional regulation. Mutations in RhoA, Rac1, or Cdc42 also result in GTP-locked proteins that can induce tumorigenesis, invasion, and metastasis in experimental models [Boettner and Van Aelst, 2002]. Numerous studies have shown that Ras, RhoA, Rac, and Cdc42 are activated by a wide range of stimuli. Lysophosphatidic acid (LPA) a major component of serum, activates both RhoA and Ras through different trimeric G proteins [Bar-Sagi and Hall, 2000]. Addition of insulin or PDGF to cultured cells leads to the rapid activation of Ras and Rac [Mackay and Hall, 1998; Vojtek and Der, 1998]. Cdc42 has been shown to be activated by the inflammatory cytokines TNFα and IL-1 [Puls et al., 1999] or bradykinin [Mackay and Hall, 1998].

Several reports have shown that Ras and Rho GTPases are involved in the regulation of COX-2 expression. Ras/Rac1/MEKK/MEK/JNK and Ras/Raf1/MEK/ERK pathways are required for PDGF or serum induced activation of the COX-2 promoter [Xie and Herschman, 1996]. Transient expression of constitutively active oncogenic mutants of Ras, Rac1, and RhoA, but not Cdc42 induces transcription of COX-2 in NIH3T3 cells [Slice et al., 2000]. Overexpression of constitutively active oncogenic H-Ras in Rat-1 fibroblasts or overexpression of constitutively active oncogenic K-Ras in rat intestinal epithelial cells increases COX-2 protein levels, and ERK activity is required for Ras-mediated induction of COX-2 [Sheng et al., 1998, 2001]. The protein levels of COX-2 in NIH3T3 fibroblasts, MDCK epithelial cells or HT-29 colon cancer cells are stimulated by overexpression of constitutively active oncogenic mutants of RhoA, Rac1, or Cdc42. However, only a dominant negative mutant of Cdc42 but not of RhoA or Rac1 decreases COX-2 protein levels in HT-29 cells [Aznar Benitah et al., 2003]. The stimulation of COX-2 expression in HT-29 cells by active RhoA, Rac1, or Cdc42 mutants depends on the transcription factor NF-κB. Overexpression of dominant negative RhoB in HCA-7 colon cancer cells reduces the protein levels of COX-2, while overexpression of constitutively active RhoB increases the levels of COX-2 [Shao et al., 2000]. Although these reports show that constitutively active mutants of Ras or Rho GTPases are capable of stimulating COX-2 expression, no evidence has been provided to show how these GTPases and their signaling pathways coordinately regulate the expression of endogenous COX-2.

In this study, we show that constitutively active oncogenic Ras and Rho coordinately stimulate COX-2 expression, whereas transient activation of wild-type Ras but not of RhoA or Rac1 mediates stimulation of COX-2 expression

in response to serum growth factors. Both ERK and JNK activity are required for serum- and oncogenic Ras-mediated COX-2 expression while only JNK links oncogenic RhoA signaling pathway to COX-2 expression.

MATERIALS AND METHODS

Materials

EZ-Detect™ Ras, Rac1, or Rho activation kits, protease inhibitor cocktail, micro BCA protein assay, Pro-Ject™ protein transfection reagent, ImmunoPure™ goat anti-mouse or goat anti-rabbit IgG (H + L)-HRP, SuperSignal West Pico or Femto chemiluminescent substrate and X-ray films were from Pierce Biotechnology (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, and Na pyruvate were from Hyclone (Logan, UT). Penicillin, streptomycin, and amphotericin B were from Invitrogen (Carlsbad, CA). Manumycin A, damnacanthal, FTI-227, toxin A, and C3 transferase were from Calbiochem EMD Biosciences (La Jolla, CA). PD98059 and SP600125 were from Alexis (San Diego, CA). TransIT-LT1 transfection reagent was from Mirus (Madison, WI). pCMV-H-Ras(G12V) plasmid and GFP mouse monoclonal antibody (Living colors™) were from BD Biosciences Clontech (Palo Alto, CA). pUSEamp-RhoA(Q63L) plasmid and phospho-Raf-1 (Ser-338) mouse monoclonal antibody were from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against phospho-MAP kinases/ERK (p42 and p44), MAP kinases/ERK (p42 and p44) and phospho-c-Jun (Ser-63) were from Cell Signaling Technology (Beverly, MA). COX-2 mouse monoclonal antibody was from BD Biosciences Pharmingen. Myc-tag mouse monoclonal antibody (9E10) and Raf-1 mouse monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin mouse monoclonal antibody (AC-15) was from Sigma-Aldrich (Saint Louis, MO).

Cell Culture

Mouse NIH3T3 fibroblasts were obtained from American type tissue collection (ATCC) and grown in DMEM supplemented with 10% FBS, 6 mM L-glutamine, 2 mM Na pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidity atmosphere of 5% CO_2. Cells were serum-starved for 24 h in DMEM supplemented with 0.2% FBS prior to stimulation with 10% FBS.

Cloning

The cDNA encoding H-Ras(G12V) or RhoA(Q63L) was amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagene) using pCMV-H-Ras(G12V) or pUSEamp-RhoA(Q63L) as a template. The amplified cDNAs were subcloned into pExpress/Myc to form N-terminal Myc tag fusions using *NdeI* and *BamHI* sites [Jakobi et al., 2002]. The sequences were verified by DNA sequence analysis and comparison to the corresponding database sequences. The Myc-tagged H-Ras or Myc-tagged RhoA was then subcloned into the bicistronic retroviral expression vector pRetroIRESgfp using *PacI* and *BamHI* sites [Jakobi et al., 2003]. The pEGFP-Rac1(Q61E) plasmid was a gift by Dr. Joe Barbieri, Medical College of Wisconsin [Krall et al., 2002].

DNA Transfection

For transient expression of active H-Ras(G12V), RhoA(Q63L), or Rac1(Q61E), NIH3T3 cells were plated in 35 mm plates at a density of 2×10^5 cells 24 h before transfection. Cells were transfected with 2 μg of pRetroIRESgfp-Myc-H-Ras(G12V), pRetroIRESgfp-Myc-RhoA(Q63L), or pEGFP-Rac1(Q61E) using 6 μl TransIT-LT1. For co-expression experiments, cells were transfected with 1 μg of each pRetroIRESgfp-Myc-H-Ras(G12V) and pRetroIRESgfp-Myc-RhoA(Q63L) or 1 μg of each pEGFP-Rac1(Q61E) and pRetroIRESgfp-Myc-RhoA(Q63L) using 6 μl of TransIT-LT1. The transfection efficiency was 20%–25%. Transfection of pRetroIRESgfp-Myc-Ras(G12V) or pRetroIRESgfp-Myc-RhoA(Q63L) into mammalian cells resulted in bicistronic expression of Myc-Ras(G12V) or Myc-RhoA(Q63L) and enhanced green fluorescent protein (EGFP). Transfection of pEGFP-Rac1(Q61E) resulted in expression of a Rac1(Q61E) fusion with EGFP at N-terminus. At 24 h after transfection, cells were incubated in DMEM supplemented with 0.2% FBS for an additional 24 h. Cells were washed once with ice-cold Tris-buffered saline (TBS) and then lysed in 200 μl ice-cold EZ-Detect™ lysis/binding/washing buffer containing a protease inhibitor cocktail and 200 μM Na othovanadate. Cell lysates were clarified by

centrifugation at 13,000*g* at 4°C for 15 min and quantified by a micro BCA assay using BSA as the standard. Aliquots of 15 μg protein were analyzed by Western blot.

Small GTPase Activation Assay

The activation of Ras, Rac1, and Rho was assayed using the EZ-Detect™ Ras, Rac1, or Rho activation kits according to manufacturer's instruction. NIH3T3 cells were plated in 100-mm plates at a density of 2×10^6 cells/plate. The next day cells were serum-starved by incubation in DMEM supplemented with 0.2% FBS for an additional 24 h. Cells were stimulated with 10% FBS for the times indicated. After treatment, cells were chilled on ice, washed once with ice-cold TBS and lysed in the ice-cold EZ-Detect™ lysis/binding/washing buffer containing a protease inhibitor cocktail and 200 μM orthovanadate (300 μl/plate). Cell lysates were clarified by centrifugation at 13,000*g* at 4°C for 15 min and quantified using micro BCA protein assay with BSA as the standard. Equal amounts of lysates (1 mg) were incubated with GST-Raf1-RBD, GTS-PAK1-PBD, or GST-Rhotekin-RBD and one SwellGel™ Immobilized Glutathione disc in a spin cup with a collection tube at 4°C for 1 h. The resin was washed three times with lysis/binding/washing buffer. Bound proteins were eluted by incubation in 50 μl 2X SDS sample buffer at 95°C for 5 min. Half (25 μl) of the sample volume was analyzed by Western blot using the antibody against Ras, Rac1, or Rho provided in the kit. The antibody against Ras is a Pan-Ras monoclonal antibody and reacts with H-, K-, and N-Ras. Rho monoclonal antibody was raised against full length RhoA and recognizes RhoA, B, and C isoforms.

Analysis of Raf-1, ERK, and c-Jun Phosphorylation/Activation and COX-2 Expression

To detect the Raf-1, ERK1/2, or c-Jun activation or COX-2 expression, cells were plated in 35-mm plates at a density of 2.5×10^5 cells. The next day, cells were serum-starved for 24 h. Cells were then treated with 10% serum for the times indicated. To inhibit ERK or JNK activity, cells were incubated with PD98059 (50 μM) or SP600125 (20 μM) for 1-h prior to serum stimulation. After serum treatment with or without the inhibitor, cells were washed with ice-cold TBS and lysed in 200 μl of ice-cold EZ-Detect™ lysis/binding/washing buffer containing a protease inhibitor cocktail and 200 μM orthovanadate. All lysates were subjected to a micro BCA protein assay using BSA as the standard. Aliquots of 15 or 20 μg proteins were analyzed by Western blot.

Treatment With Ras or Rho Inhibitor

For treatment with manumycin A or toxin A, NIH3T3 cells were plated in 100-mm plates at a density of 2×10^6 cells/plate. The next day cells, were serum-starved for an additional 24 h. Then cells were incubated with manumycin A (5 μM), FTI-277 (10 or 20 μM), or damnacanthal (10 or 20 μg/ml) for 1 h or toxin A (50 ng/ml) for 3 h. Cells were stimulated with 10% FBS for the indicated time and lysed in ice-cold EZ-Detect™ lysis/binding/washing buffer (300 μl/plate) containing a protease inhibitor cocktail and 200 μM orthovanadate. Equal amount of lysates (1 mg) were subjected to small GTPase activation assays.

For C3 treatment, C3 transferase was introduced into cells using Pro-Ject™ protein transfection reagent according to the manufacturer's instruction. Cells were plated in 100-mm plates at a density of 2×10^6 cells/plate. The next day, cells were serum-starved for 24 h. The cells were transfected with 50 μg C3 per plate using 35 μl of transfection reagent. After 3 1/2 h of incubation, cells were treated with serum at the indicated time and lysed in ice-cold EZ-Detect™ lysis/binding/washing buffer (300 μl/plate) containing a protease inhibitor cocktail and 200 μM orthovanadate. Equal amount of lysates (1 mg) were assayed for Rho activation.

Western Blot Analysis

Proteins were separated by SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA (Fraction V) in TBS at room temperature for 2 h and incubated overnight at 4°C with EZ-Detect™ anti-Ras (1:200), anti-Rac1 (1:1,000) or anti-Rho (1:500) antibody, anti-Myc-tag (9E10, 1:200), anti-EGFP (1:1,000) or anti-β-actin (1:4,000) antibody, or anti-phospho-Raf-1 (1:1,000), anti-Raf-1 (1:200), anti-phospho-MAPK/ERK (1:1,000), anti-MAPK/ERK (1:1,000), anti-phospho-c-Jun (1:1,000), anti-COX-2 (1:250) antibody diluted in 3% BSA in TBST (TBS + 0.1% Tween 20). Membrane was washed with TBST and incubated with ImmunoPure™ goat

anti-mouse or goat anti-rabbit IgG (H + L)-HRP (1:25,000 dilution in 5% non-fat dry milk in TBST) for 1 h at room temperature. Membrane was washed with TBST and the detection was performed using SuperSignal West Pico chemiluminescent substrate followed by exposure to X-ray film. For detection of phospho-c-Jun, SuperSignal West Femto chemiluminescent substrate was used. Signal intensity was quantified by exposure of the Western blot to a CCD camera followed by densitometric analysis using a FluorChem™ imaging system (Alpha Innotech).

RESULTS

Coordinate Stimulation of COX-2 Expression by Co-Expression of Constitutively Active H-Ras (G12V) and RhoA(Q63L) or Rac1(Q61E) and RhoA(Q63L)

Previous studies have shown that individual expression of constitutively active Ras or Rho small GTPases results in an increase of COX-2 expression [Sheng et al., 1998, 2001]. Here we investigate if overexpression of constitutively active Ras and Rho GTPases coordinately regulates the induction of COX-2 protein (Fig. 1). NIH3T3 cells were transfected with plasmids containing Myc-Ras(G12V), Myc-RhoA(Q63L), or EGFP-Rac1(Q61E) individually or in combination. Cells serum-starved for 24 h were used as a negative control and cells treated with serum for 3 h following serum starvation were used as a positive control. The expression of small GTPases was confirmed by probing the membrane with an anti-Myc or anti-EGFP antibody. The bands of COX-2 from the Western blot were quantified by densitometry. Transient transfection of individual constitutively active Myc-H-Ras(G12V), Myc-RhoA(Q63L), and EGFP-Rac1(Q61E) increased the protein levels of COX-2 in NIH3T3 cells as compared to serum-starved non-transfected cells. Surprisingly, co-expression of active Ras and active RhoA or active RhoA and active Rac1 coordinately stimulated the expression of COX-2. However, co-expression of active Ras and active Rac1 did not result in coordinate stimulation of COX-2 expression (data not shown). These data suggest that individual small GTPases are capable of stimulating COX-2 expression but that the coordinate activation of these small GTPases is required for full stimulation of COX-2 expression.

Kinetics of Ras, Rac1, and Rho Activation in Response to Serum Growth Factors

To understand the roles of Ras and Rho family GTPases in the regulation of COX-2 expression under physiological conditions, we examined the function relationship between the activation of these GTPases and expression of COX-2 in response to serum growth factors. Serum is a strong inducer of COX-2 expression in murine fibroblasts [Fletcher et al., 1992]. The activation of Ras and Rho family GTPases in response to serum growth factors was determined by pull-down assays in which the GTP-bound active forms of small GTPases were determined by specific binding of the active GTPase to the binding domain of effector proteins downstream of the small GTPases. The RBD (Ras binding domain) of Raf1, PBD (p21 binding domain) of PAK1 or RBD (Rho binding domain) of Rhotekin was fused to glutathione-S-transferase and used to pull-down active GTP-bound Ras, Rac1/Cdc42 or RhoA, respectively [Ren and Schwartz, 2000; Taylor et al., 2001; Benard and Bokoch, 2002]. NIH3T3 cells were serum-starved and then treated with or without 10% FBS (Fig. 2A). The lysates corresponding to each time point were also analyzed by Western blot to confirm that the total protein levels of each GTPase remained unchanged through the time course. Addition of serum resulted in an initial increase of Ras activity at 1–2 min, followed by a reduction to basal levels at 5–10 min, a second increase at 30–45 min and then returned to basal levels at 60-min. However, the initial increase of Ras activity was significantly greater than the second increase. In contrast, treatment of serum resulted in a transient increase of active RhoA, reaching a maximum activation between 1 and 2 min, and returning to basal levels at 5 min. Activation of Rac1 was detected at 2 min after addition of serum, with maximal stimulation at 5 min, sustained up to 30 min, and returned to basal levels at 60 min. At 1 min after serum stimulation the activity of Rac1 was not increased yet (data not shown). No activation of Cdc42 in response to serum stimulation was detected (data not shown). The data show that serum growth factors differentially regulate the activities of Ras, RhoA, and Rac1.

Serum growth factors activate Ras, which subsequently activates a cascade of kinases: Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK

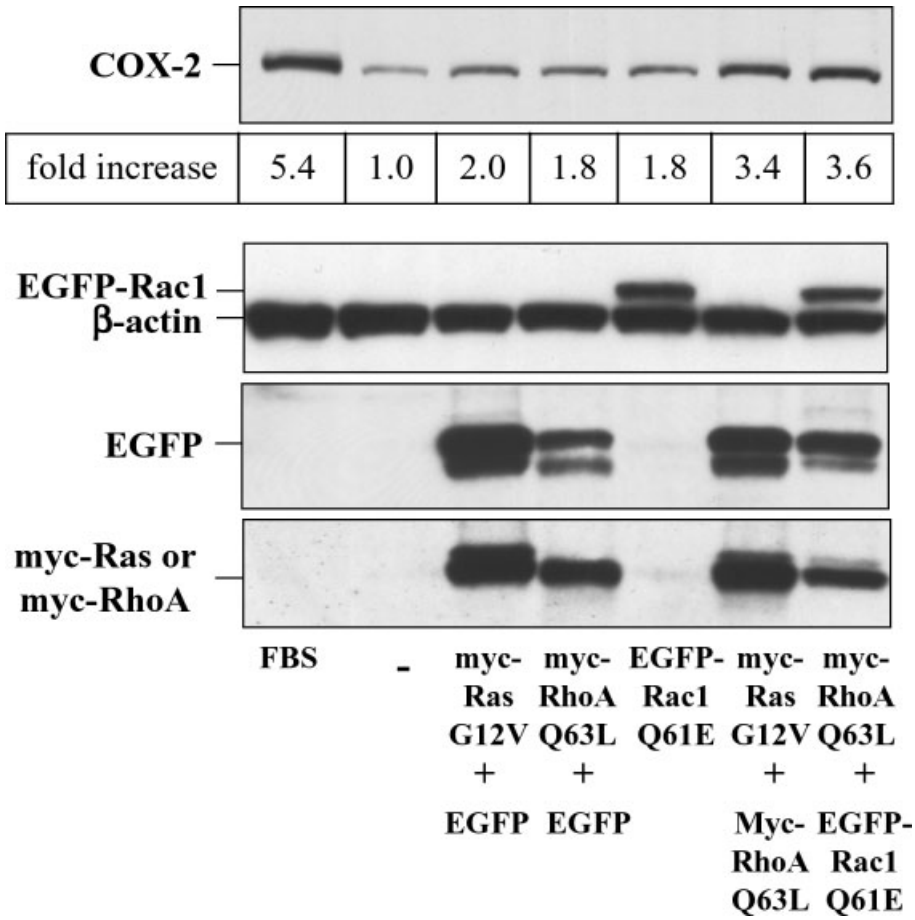


Fig. 1. Expression of constitutively active Ras(G12V), RhoA(Q63L), and Rac1(Q61E) coordinately stimulates COX-2 expression. NIH3T3 cells were transfected with pRetroIRESgfp-Myc-H-Ras(G12V), pRetroIRESgfp-Myc-RhoA(Q63L), or pEGFP-Rac1(Q61E) individually or in combination. At 24 h after transfection, cells were incubated in DMEM supplemented with 0.2% FBS for an additional 24 h. Transfection of pRetroIRESgfp-Myc-Ras(G12V) or pRetroIRESgfp-Myc-RhoA(Q63L) resulted in bicistronic expression of Myc-Ras(G12V) or Myc-RhoA(Q63L) with enhanced green fluorescent protein (EGFP). Transfection of pEGFP-Rac1(Q61E) resulted in expression of Rac1(Q61E) fusion with EGFP at the N-terminus. For a positive control, cells were stimulated with 10% FBS for 3 h following 24 h serum starvation. The lysates (15 μg) were analyzed by Western blot using an anti-COX-2 antibody. The same membrane was reprobed with an antibody against the Myc-tag to confirm the expression of Myc-Ras(G12V) and Myc-RhoA(Q63L). Subsequently, the membrane was reprobed again with an antibody against EGFP to confirm the expression of EGFP-Rac1(Q61E) and EGFP as well as with an anti-β-actin antibody to confirm the equal loading of the samples. The intensity of the COX-2 bands was determined by densitometry. The experiments were repeated twice with similar results and representative data are shown.

kinases 1 and 2), and ERK1/2. In addition to activation of the ERK1/2 pathway, serum growth factors activate the c-Jun N-terminal kinase (JNK) pathway. Activation of both ERK and JNK pathways by serum results in the stimulation of gene expression. Both ERK and JNK have been shown to be important for transcriptional stimulation of COX-2 expression in response to serum [Xie and Herschman, 1996; McGinty et al., 2000]. JNK phosphorylates and activates the transcription factor c-Jun, which is involved in transcriptional activation of the COX-2 promoter in response to serum [Xie and Herschman, 1996]. We examined activation of Raf-1, ERK1/2, and c-Jun as well as induction of COX-2 protein in response to serum (Fig. 2B). Activation of Raf-1, ERK1/2, and c-Jun was monitored by Western blot analysis of NIH3T3 cell lysates using anti-phospho-Raf1 (Ser-338), anti-phospho-ERK1/2 (Thr-202/Tyr-204), and anti-phospho-c-Jun (Ser-63) antibodies (Fig. 2B). Activation of Raf-1 was increased at 2 min after addition of serum and increased up to 60 min. Stimulation of ERK1/2 phosphorylation was elevated at 1 min after the addition of serum, with a maximal stimulation at 2–20 min, and a slow decline after 30 min. However, the levels of phosphorylated ERK1/2 at

60 min were still higher than the control (0 min). The fact that ERK activation was detected earlier than Raf-1 activation is probably due to the lower sensitivity of the anti-phospho-Raf antibody as compared to the anti-phospho-ERK1/2 antibody. Activation of the c-Jun transcription factor was detected at 2-min after addition of serum, with maximal stimulation at 10 min and declined at 30 min. The activation of p38 MAP kinase was also examined by Western blot analysis using anti-phospho-p38 antibody, and no activation of p38 was detected (data not shown). The expression of COX-2 was detected at 1 h after treatment of serum, maximal at 2–3 h and sustained up to 4 h. Our data suggest that serum-induced activation of Ras, Rho, and Rac1 correlates with activation of Raf-1, ERK, and c-Jun and stimulation of COX-2 expression.

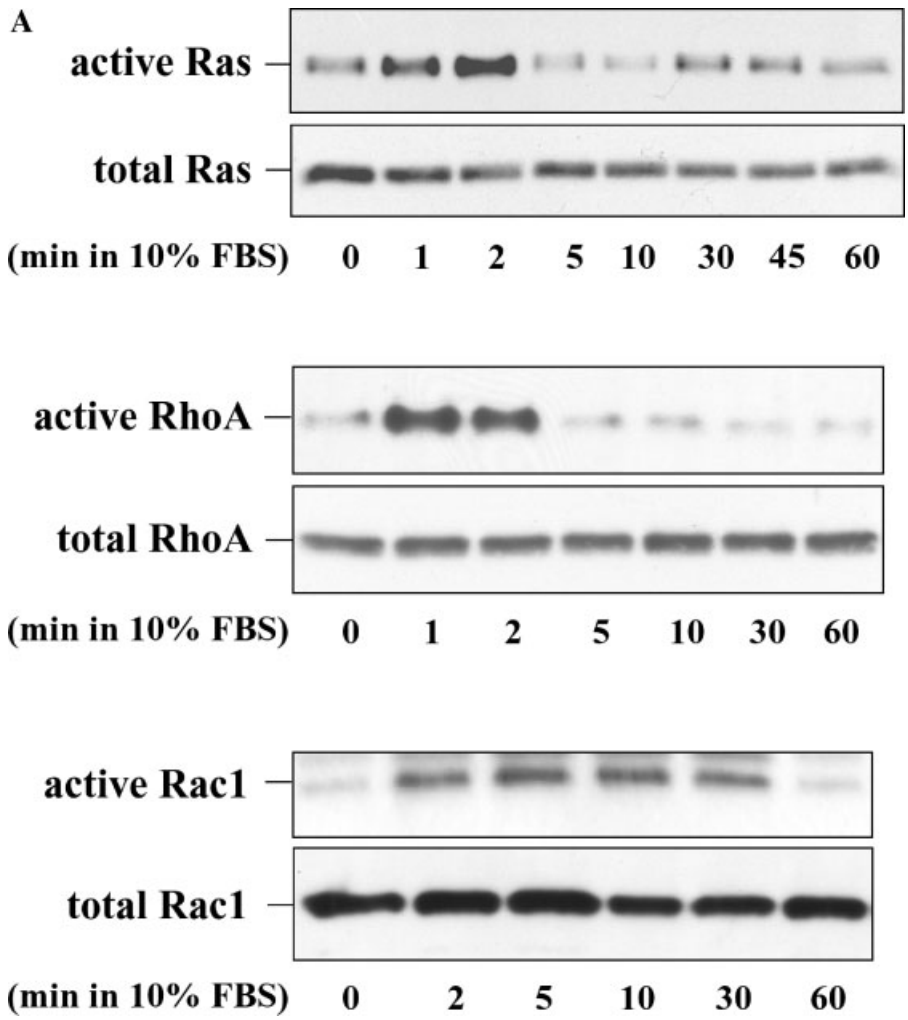


Fig. 2. Time course of activation of Ras, RhoA, and Rac1 GTPases, activation of Raf-1, ERK1/2 (p44 and p42), and c-Jun and expression of COX-2 in response to serum growth factors. **A**: Activation of Ras, RhoA, and Rac1. NIH3T3 cells, serum starved for 24 h, were stimulated with 10% FBS for the times indicated. The activity of Ras, RhoA, or Rac1 was assayed by incubation of lysate (1 mg) with GST-Raf1-PBD (**upper panel**), GST-Rhotekin-RBD (**middle panel**), or GST-PAK1-PBD (**lower panel**) in the presence of a SwellGel™ Immobilized Glutathione disc. The bound proteins were eluted and half of the pull-down sample volume was analyzed by Western blot using anti-Pan-Ras (upper panel), anti-RhoA (middle panel), or anti-Rac1 (lower panel) antibody. Lysates (15 μg) were analyzed by Western blot to verify that the total amount of each GTPase remained unchanged. The experiments were repeated three times with similar results and representative results are shown. **B**: Activation of Raf-1, ERK1/2, and c-Jun and induction of COX-2. Serum-starved NIH3T3 cells were treated with 10% FBS for the times indicated. Lysates (15 μg) were analyzed by Western blot using an antibody specific to phospho-Raf-1, phospho-ERK1/2 (p44 and p42), phospho-c-Jun, or COX-2. Typically, a non-specific band just above phospho-Raf1 in the lysate from serum-starved (0-time point) cells was detected. The membrane was reprobed with an antibody against Raf-1, ERK1/2, or β-actin. The experiments were repeated four times with similar results and representative results are shown.

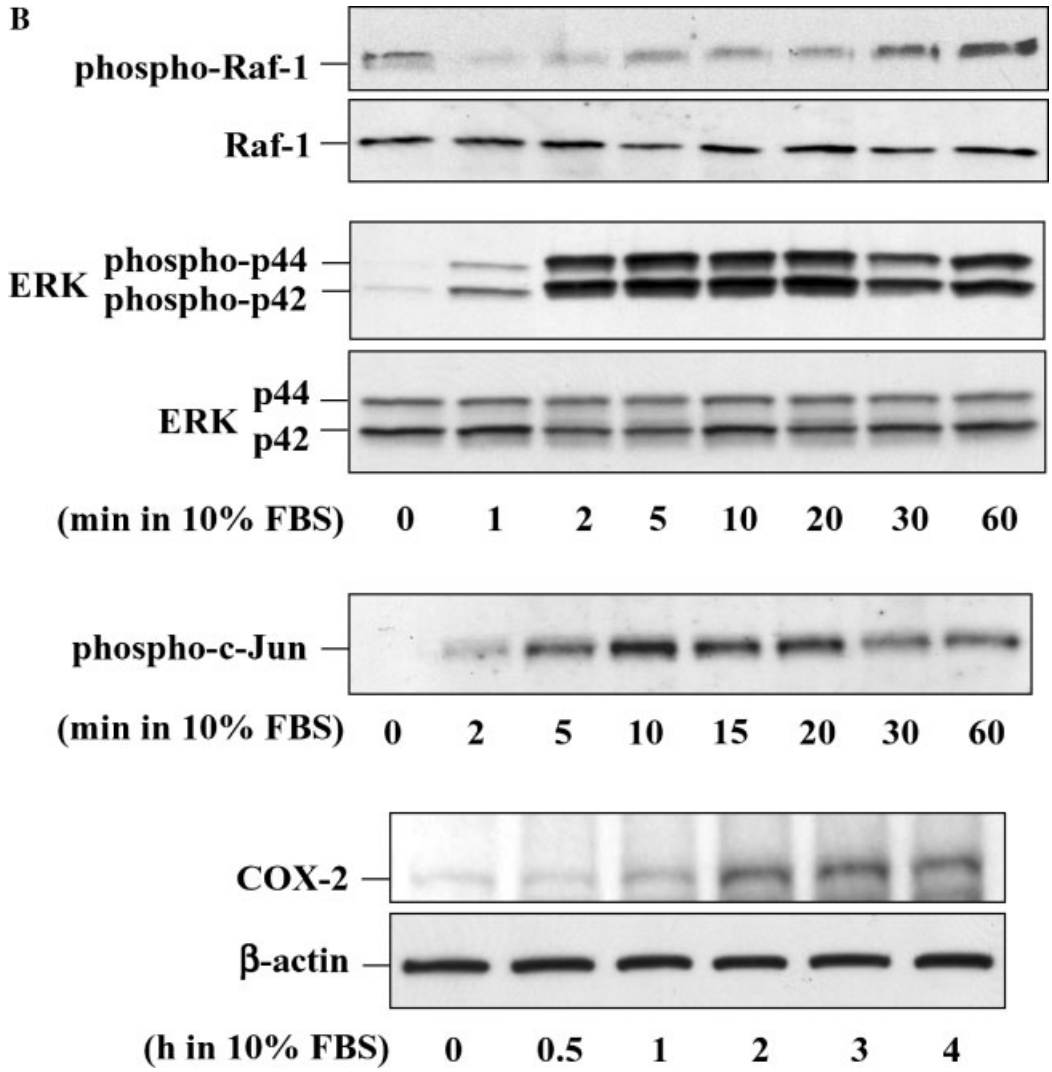
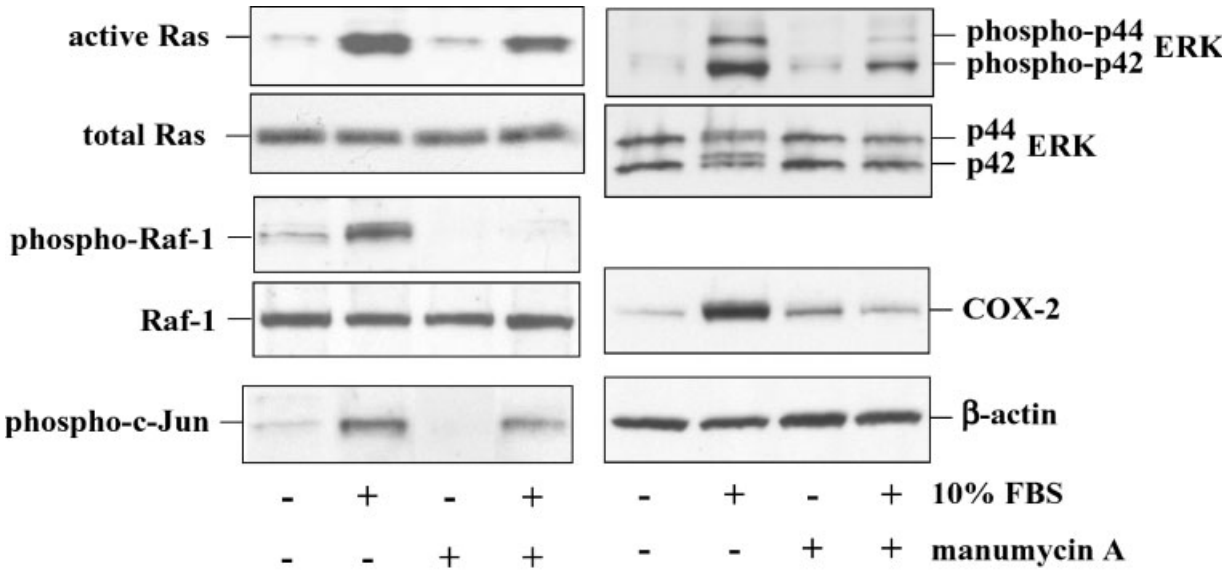


Fig. 2. (*Continued*)

Inhibition of Ras, but not Rac1 and RhoA Results in the Decrease of COX-2 Expression

Previous studies showed that expression of dominant negative Ras or Rac1 inhibits the activation of the COX-2 promoter in response to serum or PDGF [Xie and Herschman, 1996]. We found that maximal stimulation of COX-2 protein levels requires co-expression of constitutively active Ras, Rac1, and RhoA (Fig. 1). To examine whether Ras, Rac1, and/or RhoA activation is required for stimulation of COX-2 protein levels in response to serum, NIH3T3 cells were pretreated with specific inhibitors for these small GTPases. Manumycin A, a Ras functional inhibitor [Hara et al., 1993], was added to serum-starved NIH3T3 cells, followed by treatment with 10% serum. Active Ras was isolated from the lysates by incubation with GST-Raf1-RBD and detected by Western blot analysis (Fig. 3). The bands corresponding to active Ras, Raf-1, ERK1/2 and c-Jun, and COX-2 protein on the Western blots were quantified by densitometry. Pretreatment with manumycin A (5 μM) decreased the activation of Ras to 45% of control cell levels in response to serum. Furthermore, inhibition of Ras activity by manumycin A blocked the activation of Raf-1, and decreased the activation of ERK1 (p44) to 7%, ERK2 (p42) to 15%, and c-Jun to 27% and the stimulation of COX-2 expression to 11% of control cell levels in response to serum (Fig. 3). No further inhibition of Ras was observed upon increasing the dose to 10 μM (data not shown), suggesting that a small portion of cells were resistant to the treatment of manumycin A.



active Ras	phospho-Raf-1	phospho-ERKp44	phospho-ERKp42	phospho-c-Jun	COX-2 levels
45	0	7	15	27	11

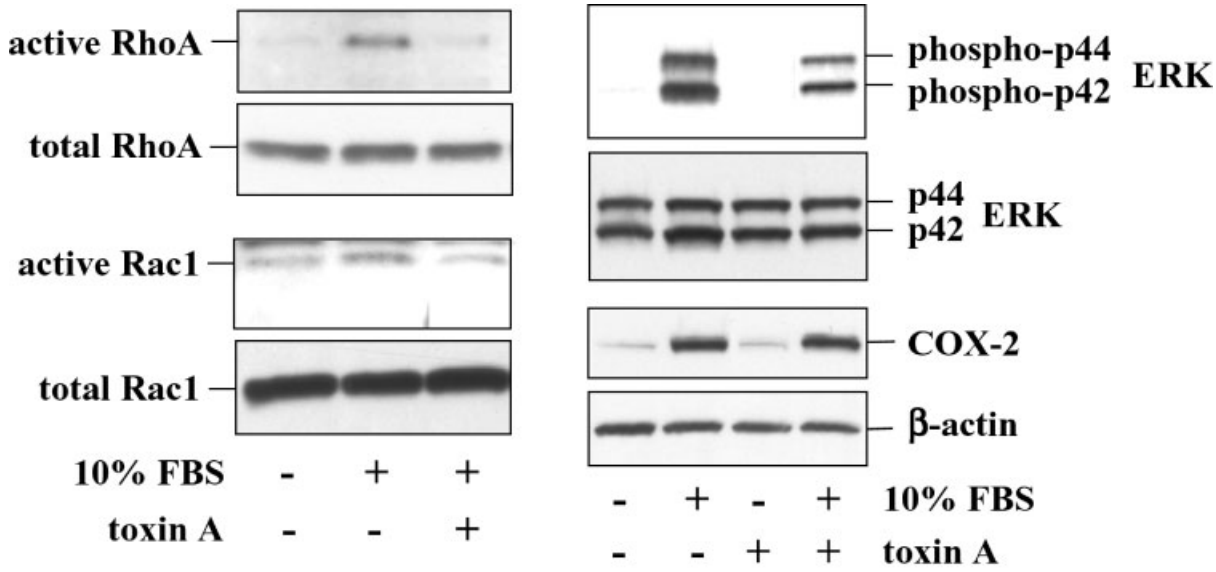
Inhibition by manumycin (in percentages of 10% FBS control)

Fig. 3. Blocking Ras activity by manumycin A results in inhibition of Raf-1, c-Jun, and ERK1/2 activation, and inhibition of COX-2 expression. Serum-starved NIH3T3 cells were pretreated with or without manumycin A (5 μM) for 1 h at 37°C, followed by stimulation with 10% FBS for 1 min to assay Ras activity, for 5 min to examine activation of Raf-1 and ERK1/2, for 10 min to examine activation of c-Jun, or for 3 h to analyze expression of COX-2. To assay Ras activity, 1 mg of lysate was incubated with GST-Raf1-RBD in the presence of SwellGel™ Immobilized Glutathione disc. Half of the pull-down sample volume and lysates (15 μg) were analyzed by Western blot using an anti-Pan-Ras antibody. For phospho-Raf-1, phospho-c-Jun phospho-ERK1/2, and COX-2, 20 μg of the lysates were analyzed by Western blot using an antibody specific for phospho-Raf-1, phospho-c-Jun phospho-ERK1/2, or COX-2. The membranes were reprobed with anti-Raf-1, anti-ERK1/2, or anti-β-actin antibody. The intensities of bands corresponding to active Ras, phospho-Raf1, phospho-ERK1, and 2, phospho-c-Jun, and COX-2 were determined by densitometry. The experiments were repeated three times with similar results and representative results are shown.

Moreover, approximately 50% of cells were detached from the plates in the presence of 10 μM of manumycin A. A similar inhibitory effect on Ras, Raf-1, ERK1/2, and c-Jun activation and COX-2 expression was observed when Ras inhibitors, FTI-277 (10 or 20 μM) [Lerner et al., 1995] or damnacanthal (10 or 20 μg/ml) [Hiramatsu et al., 1993], were used (data not shown).

Toxin A or toxin B from *Clostridium difficile* glycosylates and inactivates Rho-like small GTPases, such as Rho, Rac, and Cdc42 [Just et al., 1995a,b]. Serum-starved NIH3T3 cells were treated with toxin A (50 ng/ml), and then stimulated with 10% serum. The amount of active RhoA or Rac1 was monitored by incubation of the lysates with GST-Rhotekin-RBD or GST-PAK1-PBD, followed by Western blot analysis using anti-RhoA or anti-Rac1 antibody (Fig. 4). Pretreatment of NIH3T3 cells with toxin A completely blocked the activation of RhoA and Rac1. The bands of phosphorylated ERK1/2 and COX-2 from the Western blots were quantified by densitometry. The serum-induced activation of ERK decreased to 28% for ERK1 (p44) and 43% for ERK2 (p42) in the presence of toxin A. In contrast, pretreatment with toxin A had little effect on the induction of COX-2 proteins in response to serum.

C3 transferase, which ADP ribosylates and inactivates RhoA but not Rac1 [Hill et al., 1995] was transfected to NIH3T3 cells (Fig. 5). A concentration of 10 μg/ml of C3 transferase was efficient to inhibit the activation of Rho in



active RhoA	active Rac1	phospho-ERKp44	phospho-ERKp42	COX-2 levels
0.5	1	28	43	100

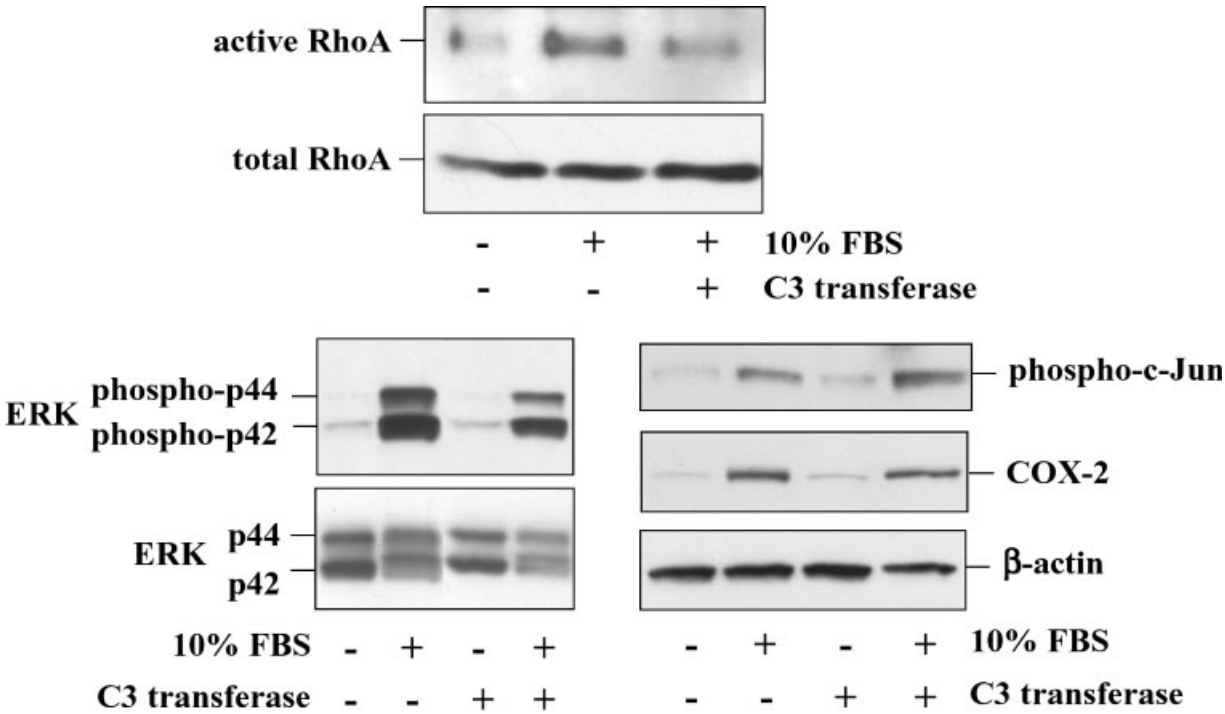
Inhibition by toxin A (in percentages of 10% FBS control)

Fig. 4. Effects of toxin A on activation of RhoA, Rac1, and ERK1/2, and induction of COX-2. Serum-starved NIH3T3 cells were preincubated with or without toxin A (50 ng/ml) for 3 h, then treated with 10% FBS for 2 min to assay the activation of RhoA, for 5 min to examine the activation of Rac1 and ERK1/2, or for 3 h to analyze the expression of COX-2. Serum-starved cells without treatment were used as controls for basal levels. To assay RhoA or Rac1 activity, 1 mg of the lysate was incubated with GST-Rhotekin-RBD or GST-PAK1-PBD in the presence of a Swell-Gel™ Immobilized Glutathione disc. Half of the pull-down sample volume and lysates (15 μg) were analyzed by Western blot using anti-RhoA or anti-Rac1 antibody. For phospho-ERK1/2 and COX-2, 20 μg of lysates were analyzed by Western blot using antibody specific for phospho-ERK1/2 or COX-2. The membranes were reprobed with anti-ERK1/2 or anti-β-actin antibody. The intensities of bands corresponding to active RhoA or Rac1, phospho-ERK1, and 2 and COX-2 were determined by densitometry. The experiments were repeated three times with similar results and representative results are shown.

response to serum. C3 treatment decreased activation of ERK1 (p44) to 43% and ERK2 (p42) to 55% of control cell levels. However, C3 treatment had no significant effect on the induction of COX-2 expression or c-Jun activation (Fig. 5). Since the inhibitory effect of C3 on ERK1/2 activation was less than by toxin A treatment, it is suggested that active Rac1 and RhoA are required for ERK1/2 activation in response to serum. In contrast, inhibition of both Rac1 and RhoA did not affect serum stimulation of COX-2 expression.

Role of the ERK and JNK Pathways on Induction of COX-2 Expression

A specific inhibitor for MEK (PD98059) or JNK (SP600125) [Bennett et al., 2001] was used to determine direct correlation between activation of ERK1/2 and c-Jun and stimulation of COX-2 expression in response to serum stimulation or transient expression of constitutively active Ras or RhoA (Fig. 6). As shown in Figure 6A, PD98059 treatment partially blocked serum induction of COX-2 protein, whereas SP600125 treatment had a more potent inhibitory effect on serum-induced COX-2 expression than PD98059. Moreover, treatment with both inhibitors resulted in a cooperative inhibitory effect on COX-2 expression. The membrane was reprobed with an anti-phospho-c-Jun antibody to confirm that phosphorylation and activation of c-Jun was stimulated by serum and inhibited by treatment with JNK inhibitor (SP600125) but not by MEK inhibitor (PD98059). Reprobing



active RhoA	phospho-ERKp44	phospho-ERKp42	phospho-c-Jun	COX-2 levels
18	43	55	100	95

Inhibition by C3 transferase (in percentages of 10% FBS control)

Fig. 5. Inhibition of RhoA by C3 transferase results in decreased ERK1/2 activation but had no significant effect on COX-2 expression. Quiescent NIH3T3 cells were transfected with or without C3 transferase (10 μg/ml) using Pro-Ject™ protein transfection reagent. Three hours post transfection, cells were treated with 10% FBS for 2 min to assay RhoA activity, for 5 min to examine activation of ERK1/2, for 10 min to examine activation of c-Jun, or for 3 h to analyze COX-2 expression. To assay RhoA activity, 1 mg of the lysate was incubated with GST-Rhotekin-RBD in the presence of a SwellGel™ immobilized glutathione disc. Half of the pull-down sample volume and lysates (15 μg) were analyzed by Western blot using anti-Rho antibody. For phospho-ERK1/2 and COX-2, 20 μg of the lysates were analyzed by Western blot using antibody specific for phospho-ERK1/2, phospho-c-Jun, or COX-2. The membranes were reprobed with anti-ERK1/2 or anti-β-actin antibody. The intensities of bands corresponding to active RhoA, phospho-ERK1, and 2, phospho-c-Jun, and COX-2 were determined by densitometry. The experiments were repeated three times with similar results and representative results are shown.

with an anti-phospho-ERK1/2 antibody showed that treatment with PD98059 blocks the activation of ERK1/2. However, treatment with SP600125 resulted in elevated phosphorylation of ERK, but had no effect on stimulation of COX-2 expression. The results suggest that ERK1/2 and JNK are involved in the stimulation of COX-2 protein levels in response to serum and agree with the previous finding that serum induction of the COX-2 promoter transcription requires activation of both MEKK1/JNK and MEK/ERK signal transduction pathways [Xie and Herschman, 1996].

Expression of constitutively active oncogenic Ras or RhoA resulted in elevated phosphorylation of c-Jun and ERK1/2 (Fig. 6B). Treatment with PD98059 slightly reduced oncogenic Ras-induced COX-2 expression but had no significant effect on oncogenic RhoA-induced COX-2 expression (Fig. 6B). In contrast, SP600125 significantly blocked both oncogenic Ras and RhoA-induced COX-2 expression. Activation of

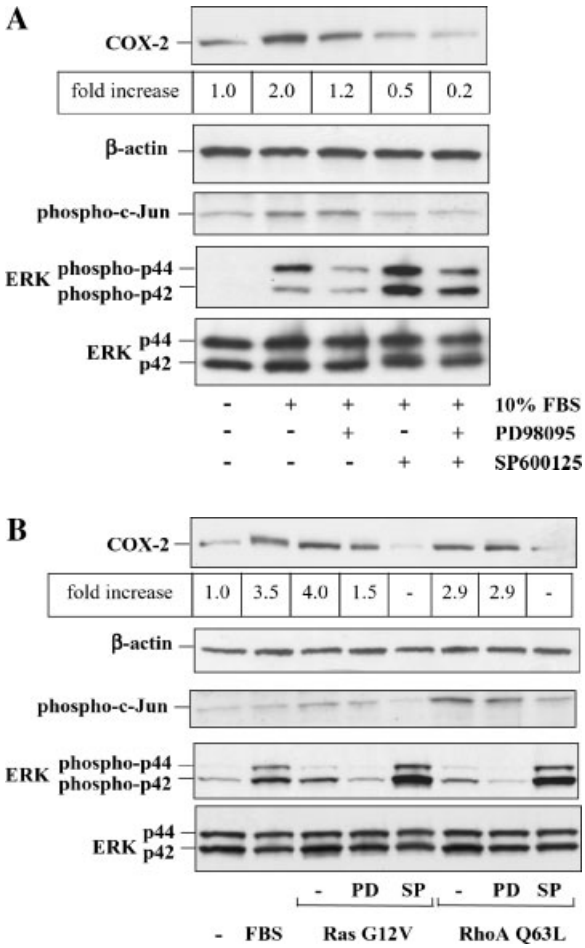


Fig. 6. ERK1/2 and JNK are required for serum or oncogenic Ras induction of COX-2 whereas JNK is involved in oncogenic RhoA induction of COX-2. **A**: PD98059 (50 μM) or SP600125 (20 μM) was added to serum-starved NIH3T3 cells for 1 h prior to treatment with 10% FBS for 3 h. The lysates (15 μg) were analyzed by Western blot using anti-COX-2, anti-phospho-c-Jun, or anti-phospho-ERK1/2 antibody. The membrane was reprobed with an anti-ERK1/2 antibody or anti-β-actin antibody. **B**: NIH3T3 cells were transiently transfected with pRetroIRESgfp-Myc-Ras(G12V) or pRetroIRESgfp-Myc-RhoA(Q63L). At 24 h after transfection, PD98059 (50 μM) or SP600125 (20 μM) was added to the cells in DMDM supplemented with 0.2% FBS for an additional 24 h. Serum-starved NIH3T3 cells treated with 10% FBS for 3 h served as a positive control. The lysates were analyzed by Western blot using anti-COX-2, anti-phospho-c-Jun, or anti-phospho-ERK1/2 antibody. The membrane was reprobed with an anti-ERK1/2 antibody or anti-β-actin antibody. The intensity of the COX-2 bands was determined by densitometry and the fold increase is tabulated. The experiments were repeated twice with similar results and representative results are shown.

transcription factor c-Jun by expression of constitutively active oncogenic Ras or RhoA was inhibited by SP600125 but not by PD58095. Phosphorylation of ERK1/2 stimulated by expression of oncogenic Ras or RhoA was inhibited by PD98059, but was further elevated by treatment with SP600125. However, elevated phosphorylation of ERK1/2 as a result of SP600125 treatment had no significant effect on stimulation of COX-2 expression. Our data suggest that the JNK pathway plays a key role in mediating oncogenic Ras or RhoA signaling in induction of COX-2 expression.

DISCUSSION

It is well established that the Ras and Rho families of small GTPases are involved in the regulation of COX-2 expression. The levels of COX-2 protein are dramatically increased in conditionally H-Ras(V12)-transformed Rat-1 cells or K-Ras(V12)-transformed rat intestinal epithelial cells [Sheng et al., 1998, 2001]. Overexpression of constitutively active RhoA, Rac1, or Cdc42 induces COX-2 expression in NIH3T3 cells, MDCK epithelial cells and HT29 colon carcinoma cells [Aznar Benitah et al., 2003]. Stable transfection of dominant negative RhoB decreases the levels of COX-2 in HCA-7 and LS-174 colon carcinoma cells in which COX-2 is constitutively expressed [Shao et al., 2000]. In contrast, expression of constitutively active RhoB further increased COX-2 expression in both HCA-7 and LS-174 cells [Shao et al., 2000]. Here we show that individual expression of constitutively active H-Ras, RhoA, or Rac1 results in an increase of COX-2 expression, whereas co-expression of constitutively active RhoA with either constitutively active Ras or Rac1 coordinately stimulates COX-2 expression. Therefore, our data suggest that maximal stimulation of COX-2 expression requires coordinate oncogenic activation of Ras, RhoA, and Rac1. It has been suggested that Ras and Rac signaling activate the COX-2 promoter through a common CRE/ATF element, whereas RhoA transactivates the COX-2 promoter independent of CRE/ATF [Slice et al., 2000]. Thus, COX-2 can be a target gene to integrate signals downstream of Ras and Rho pathways.

COX-2 is known to be expressed as an immediate-early gene in response to a variety of extracellular stimuli [Dubois et al., 1998]. To understand the functional relationship between induction of COX-2 expression and activation of Ras and Rho GTPases in response to physiological stimuli, we examined the activation of these GTPases by serum stimulation and the effects of specific inhibitors of these GTPases on COX-2 expression and ERK activation. Our

data show that the activities of Ras, Rac1, and RhoA are regulated differentially in response to serum. Serum induces activation of Ras as early as at 1–2 min, followed by a second smaller increase at 30–45 min. This is in agreement with a similar study described by Foschi et al. [1997] using endothelin-1. In contrast, the activation of RhoA is transient and detected at 1–2-min after addition of serum while Rac1 activation is at 2 min and sustained up to 30 min. The results of activation of RhoA and Rac1 are also in agreement with previous studies [Ren et al., 1999; del Pozo et al., 2000]. Treatment with manumycin A, an inhibitor of Ras farnesylation, blocks the stimulation of COX-2 expression induced by serum. In contrast, treatment with toxin A, a general Rho family inhibitor, or C3 transferase, a specific Rho inhibitor, has no significant inhibitory effects on serum-induced COX-2 expression. Therefore, our data suggest that Ras is the main regulator of COX-2 expression in response to serum growth factors. Although manumycin A is considered as a Ras functional inhibitor, it has also been suggested to block other farnesylated proteins [Cox and Der, 1997; Lebowitz and Prendergast, 1998]. Therefore, further experiments using dominant negative Ras are necessary to confirm the data from the experiments with manymycin A. Expression of dominant negative Rac1 has been shown to block the serum-induced activation of the COX-2 promoter [Xie and Herschman, 1996]. In contrast, dominant negative Rac1 had no effect on the protein levels of COX-2 in HT-29 colon cancer cells [Aznar Benitah et al., 2003]. Here we show that treatment with toxin A results in inhibition of both Rac1 and RhoA activation but has little inhibitory effects on COX-2 induction in response to serum. COX-2 expression is regulated at the levels of transcription [Xie and Herschman, 1996], post-transcription [Dixon et al., 2000], and degradation [Shao et al., 2000]. The induction of COX-2 in Rat-1 cells by H-Ras(V12) or in intestinal epithelial cells by K-Ras(V12) is mediated by an increase of COX-2 transcription as well as the stability of COX-2 mRNA [Sheng et al., 1998, 2001]. Our data show that serum stimulates the activation of Ras, which possibly leads to an increase of COX-2 transcription and stability of COX-2 mRNA. Therefore, blocking Ras activity appears to result in a complete inhibition of COX-2 induction in response to serum by inhibiting at the levels of transcription and post transcription.

The kinetics show that Raf-1 activation in response to serum is sustained up to 60-min even Ras is no longer active at that time. Sustained activation of Raf-1 could result from a lack of phosphatase to dephosphorylate/inactivate Raf-1 during that time course [Raabe and Rapp, 2003]. Activation of ERK 1/2 by serum stimulation was detected at 1-min, with a maximal stimulation between 2 and 20 min and slowly decline after 30 min, but remains above the control level for 60 min. The activity of ERK is reversibly regulated by phosphorylation and dephosphorylation. Although Ras is no longer active, phosphorylation of ERK remains elevated at 60 min after serum addition. These may be due to a lack of MAPK phosphatases (MKP) for ERK during that time course [Camps et al., 2000]. Analysis of the phosphorylation/activation of c-Jun following serum stimulation showed that maximum activation occurs at 10 min and starts to decline at 30 min, but remains above the control level for 60 min. Pretreatment of cells with manumycin A, a Ras functional inhibitor, results in significant reduction of serum-induced Raf-1, ERK1/2, and c-Jun activation. The Rho family inhibitor, toxin A, also partially prevents ERK1/2 activation in response to serum. Although the activation of ERK1/2 in response to serum appears to be mainly mediated by Ras, RhoA, and Rac1 also play roles in the serum-induced activation of ERK1/2. The main pathway to activate ERK1/2 is through Raf and MEK; Raf is the downstream effector of Ras. However, Rac could stimulate ERK1/2 activation through its downstream effector p21 activated kinase (PAK). PAKs have been shown to phosphorylate both Raf and MEK and phosphorylation by PAKs appears to be required for activation of Raf and MEK [Bar-Sagi and Hall, 2000]. Therefore, regulation of ERK1/2 activation by serum involves cross-talk between Ras- and Rac-mediated signaling pathways. We attempted to analyze the activation state of PAK in response to serum using an antibody specific to PAK phosphorylated at a threonine residue in the activation loop. However, the data were not conclusive, probably because the antibody is not sensitive enough to detect endogenous phospho-PAK. So far no evidence of a direct link between RhoA and ERK1/2 activation in response to serum growth factors has been reported. The results

presented here are the first data to show that ERK1/2 activation by serum is decreased when cells are transfected with the specific Rho inhibitor, C3 transferase. Therefore, RhoA signaling appears to provide a novel mechanism leading to ERK1/2 activation in response to serum, which is parallel to the Ras- and Rac-mediated pathways.

Ras/Rac1/MEKK/MEK/JNK and Ras/Raf1/MEK/ERK pathways were shown to be required for PDGF or serum induced activation of the COX-2 promoter [Xie and Herschman, 1996]. Furthermore, MEK/ERK activity is essential for Ras-mediated induction of COX-2 [Sheng et al., 1998]. We show that treatment of NIH3T3 cells with the MEK inhibitor PD98059 partially reduces the protein levels of COX-2 induced by serum. In contrast, the JNK inhibitor SP600125 significantly blocks COX-2 levels induced by serum. Moreover, treatment with both inhibitors results in a cooperative inhibitory effect on serum-induced COX-2 expression, suggesting both ERK1/2 and JNK signaling pathways are required for serum-induced COX-2 expression. Expression of constitutively active oncogenic Ras or RhoA stimulates expression of COX-2 as well as phosphorylation/activation of ERK1/2 and c-Jun. Treatment with the MEK inhibitor (PD98059) partially reduces the protein levels of COX-2 induced by transient expression of active Ras, but has no significant effect on protein levels of COX-2 induced by expression of constitutively active oncogenic RhoA. In contrast, treatment with the JNK inhibitor (SP600125) significantly inhibits protein levels of COX-2 induced by expression of constitutively active oncogenic Ras or RhoA. Therefore, our data suggest that both ERK1/2 and JNK pathways are involved in mediating signaling from oncogenic Ras and serum growth factors to induce COX-2 expression. We have shown that Ras is the main small GTPase to mediate signals from serum growth factors to stimulate ERK1/2 and JNK pathways and COX-2 expression. Therefore, serum growth factors appear to transiently activate Ras, which in turn activates ERK1/2 and JNK pathways and subsequently leads to stimulation of COX-2 expression.

Transient activation of RhoA in response to serum appears not to be involved in activation of c-Jun and stimulation of COX-2 expression in response to serum, whereas oncogenic activation of RhoA stimulates c-Jun phosphorylation and COX-2 expression. Furthermore, our data provide the first evidence that JNK links oncogenic RhoA signaling to COX-2 expression. A previous report showed that Rho kinase (ROCK) is involved in promoting COX-2 expression induced by oncogenic RhoA [Aznar Benitah et al., 2003]. A recent study suggests that the RhoA-ROCK pathway activates JNK, which then phosphorylates c-Jun and ATF2 when bound to the c-Jun promoter, thus stimulating c-Jun expression [Marinissen et al., 2004]. Therefore, JNK appears to be either parallel to or downstream of ROCK signaling to connect RhoA signaling and COX-2 expression.

COX-2 is overexpressed in cancers of the colon [Eberhart et al., 1994], gastric [Ristimaki et al., 1997], prostate [Yoshimura et al., 2000], and breast [Half et al., 2002], and chronic inhibition of COX-2 activity is associated with chemopreventive effects on colon cancer [Hawk et al., 2002]. However, the identification of the pathways and regulatory elements that lead to COX-2 expression in cancer development largely remain unknown. Our data provide the first evidence to suggest that oncogenic activation of Ras, Rac1, and RhoA coordinately stimulate COX-2 expression. Although stimulation with serum growth factors result in transient activation of Ras, Rho, and Rac1, Ras is the main regulator mediating serum growth factor signaling to stimulate COX-2 expression through activation of ERK1/2 and JNK pathways. Both ERK and JNK pathways are involved in oncogenic Ras-induced COX-2 expression, whereas the JNK pathway plays a key role in linking oncogenic RhoA signaling pathways to COX-2 expression.

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