Induction of Cytosolic Phospholipase A2 by Oncogenic Ras Is Mediated through the JNK and ERK Pathways in Rat Epithelial Cells*

Mutations in ras genes have been detected with high frequency in nonsmall cell lung cancer cells (NSCLC) and contribute to transformed growth of these cells. It has previously been shown that expression of oncogenic forms of Ras in these cells is associated with elevated expression of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2), resulting in high constitutive levels of prostaglandin production. To determine whether expression of constitutively active Ras is sufficient to induce expression of these enzymes in nontransformed cells, normal lung epithelial cells were transfected with H-Ras. Stable expression of H-Ras increased expression of cPLA2 and COX-2 protein. Transient transfection with H-Ras increased promoter activity for both enzymes. H-Ras expression also activated all three families of MAP kinase: ERKs, JNKs, and p38 MAP kinase. Expression of constitutively active Raf did not increase either cPLA2 or COX-2 promoter activity, but inhibition of the ERK pathway with pharmacological agents or expression of dominant negative ERK partially blocked the H-Ras-mediated induction of cPLA2 promoter activity. Expression of dominant negative JNK kinases decreased cPLA2 promoter activity in NSCLC cell lines and inhibited H-Ras-mediated induction in normal epithelial cells, whereas expression of constructs encoding constitutively active JNKs increased promoter activity. Inhibition of p38 MAP kinase or NF-κB had no effect on cPLA2 expression. Truncational analysis revealed that the region of the cPLA2 promoter from −58 to +12 contained sufficient elements to mediate H-Ras induction. We conclude that expression of oncogenic forms of Ras directly increases cPLA2 expression in normal epithelial cells through activation of the JNK and ERK pathways.

Gain of function mutations in ras genes have been detected in a variety of human tumors, including colon, prostate, and lung. Activating mutations in Ras are associated with nonsmall cell lung cancer (NSCLC),1 occurring in ~30% of adenocarcinomas, and just under 10% of other NSCLC types (1). It is presumed that expression of these forms of Ras, which lack intrinsic GTPase activity, mediate transformation by constitutively activating downstream effector pathways. We recently reported that expression of oncogenic forms of Ras was associated with increased expression of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2) in a panel of NSCLC lines (2). The signaling pathways that mediate induction of cPLA2 and COX-2 in these cells are undefined.

cPLA2 is the major intracellular form of PLA2, which selectively hydrolyzes membrane phospholipids at the sn-2 position and is the rate-limiting enzyme in the regulated release of arachidonic acid (AA) (3, 4). Free AA is metabolized through three major pathways to produce eicosanoids. Cyclooxygenases (COX) convert AA to prostaglandins and thromboxane; lipooxygenases produce leukotrienes and hydroxyeicosatetraenoic acids and cytochrome P-450 epoxygenase produces epoxycyclooctatetraenoic acids. Two forms of cyclooxygenase have been identified (5). COX-1 appears to be constitutively expressed in most cell types and is associated with maintenance of vascular tone. COX-2, first identified as an immediate early response gene (6), is induced in response to mitogenic stimuli and associated with inflammation. High levels of cPLA2 and COX-2 expression result in constitutively high levels of prostaglandin production by NSCLC (7, 8). We (2) and others (9–11) have demonstrated that nonsteroidal anti-inflammatory agents, which are inhibitors of eicosanoid production, blocked the transformed growth of NSCLC expressing Ras mutations. Because these agents do not inhibit the growth of most nontransformed cells, it suggests that this pathway plays a critical role in transformed growth.

Ras has been demonstrated to couple to multiple effector systems, resulting in activation of distinct physiologic responses (see Ref. 12 for review). These pathways regulate cell proliferation as well as changes in the cytoskeleton. An important class of Ras effectors is the mitogen-activated protein kinase (MAP kinase) family. The "classic" Ras-mediated pathway involves binding and activation of the serine/threonine kinase Raf-1, which in turn activates the dual specificity kinase MEK, resulting in activation of the extracellular signal-regulated kinases (ERKs) (13). ERKs phosphorylate a number of target proteins, including transcription factors and intracellular enzymes (14). Expression of constitutively active forms of Ras also can lead to increased activity of the other members of

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§ The abbreviations used are: NSCLC, nonsmall cell lung cancer; cPLA2, cytosolic phospholipase A2; COX-2, cyclooxygenase-2, JNK, c-Jun amino-terminal kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; AA, arachidonic acid; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; kb, kilobase(s); bp, base pair(s); DN, dominant negative; CSF, colony-stimulating factor.

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the MAP kinase family: the stress-activated protein kinase/c-Jun amino-terminal kinases (JNKs) and the p38 family of MAP kinase (15, 16). The physiologic roles of JNKs and p38 MAP kinase appear to be complex and may depend on the context in which these enzymes are activated. Activation in response to cell stresses or growth factors is mediated through specific upstream kinases: MKK4/7 for JNKs (17, 18) and MKK3/6 for p38 MAP kinase (19, 20). Both JNKs and p38 MAP kinase have been shown to phosphorylate transcription factors, including c-Jun, ATF-2, and members of the ets family including Elk-1 (21).

Multiple additional Ras effectors have been described. Ras activation also leads to stimulation of phosphatidylinositol 3-kinase in some cell types (22) and subsequent activation of the serine/threonine kinase Akt (23). Activation of Akt may be critical for cell survival, possibly through inhibition of apoptotic pathways (24, 25). Expression of Ras mutants containing mutations in the effector domains suggests that activation of multiple effector pathways is required for the full physiologic response to activated Ras (26).

Although studies in NSCLC cell lines have implicated a role for Ras in induction of cPLA2, these cells contain large number of mutations and aberrations in signaling pathways, making it difficult to define the critical molecular pathways regulating cPLA2 expression. We have therefore sought to examine the effects of constitutively active forms of Ras on cPLA2 expression in an untransformed lung epithelial cell line. In this study we report that expression of H-Ras is sufficient to mediate induction of cPLA2 expression in RL-65 cells, a neonatal, untransformed, immortalized rat epithelial cell line (27), and have begun to define the downstream effector pathways.

MATERIALS AND METHODS

Reagents and Constructs—The cPLA2 reporter construct contains 2.4 kb of a 5′ region ligated into the promoterless luciferase vector PA3lac (28). The COX-2 promoter ligated to a promoterless luciferase vector was a gift of Dr. Harvey Herschman (UCLA Medical Center, Los Angeles, CA). The Elk-Gal4, Jun-Gal4, and ATF-2-Gal4 plasmids encode the DNA-binding domain of Gal4 fused to the activation domain of Elk-1, c-Jun, and ATF-2, respectively. The UAS-luc plasmid contains the DNA-binding domain of Gal4 fused to the activation domain of Elk-1, c-Jun, and ATF-2, and members of the ets family including Elk-1 (21).

Based on our studies in NSCLC, we sought to determine whether expression of constitutively active forms of Ras is sufficient to induce cPLA2 in normal lung epithelial cells. We therefore examined the effects of H-Ras expression in a normal lung epithelial cell line (RL-65). RL-65 cells, derived from rat lung, display an epithelial morphology (27) and do not form colonies in soft agar. Stable cell lines expressing H-Ras were obtained by retroviral infection of RL-65 cells and selection in G-418 as described previously (35). Clones were selected by immunoblotting with anti-H-Ras antibodies.

Enzyme Assays and Immunoblotting—Measurements of cPLA2 activity were performed as described previously (36). Briefly, cell were scraped into a homogenization buffer containing 50 mM Hepes, pH 7.5, 1 mM EDTA, 2 mM MgCl2, 10 μM sodium orthovanadate, and a mixture of protease inhibitors using a Dounce homogenizer. Extracts were centrifuged at 100,000 × g for 1 h, and high speed supernatants were matched for protein. Activity was determined using [14C]arachidonoylphosphatidylcholine in the presence of 4 mM Ca2+. Results are expressed as picomoles of arachidonic acid released/mg of protein. For immunoblotting, cells were lysed in buffer containing 50 mM β-glycerophosphate, pH 7.2, 1 mM EDTA, 2 mM MgCl2, 100 μM sodium vanadate, 0.5% Triton X-100. Extracts were centrifuged at 10,000 × g, and supernatants were matched for protein. Extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes. Blots were probed with either a monoclonal antibody against cPLA2 or a rabbit polyclonal antibody against COX-2. Blots were visualized using the ECL system from Amersham Pharmacia Biotech (Arlington Heights, IL).

For measurement of ERK activity, cells were transiently transfected with an expression plasmid encoding HA-tagged ERK-1, along with either H-Ras, BxB-Raf, or empty vector. After 48 h, cells were washed with ice-cold phosphate-buffered saline, lysed in buffer (50 mM β-glycerophosphate, pH 7.2, 0.5% Triton X-100, 5 mM EDTA, 100 μM sodium orthovanadate, 1 mM dithiothreitol, 2 mM MgCl2, 0.06 unit of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 2 μM leupeptin). Insoluble material was pelleted by centrifugation (10 min, 14,000 × g), and supernatant was matched for protein. ERKs were immunoprecipitated by incubating for 2 h with anti-HA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and protein G-Sepharose beads. The beads were washed three times in lysis buffer, and kinase activity was determined using epidermal growth factor-receptor peptide as described previously (35).

RESULTS

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The COX-2 promoter ligated to a promoterless luciferase vector containing 4 m M Ca2+ (29, 30). The dominant negative form of JNK3 (33) or JNK2. The dominant negative form of ERK-1 contained similar Lys to Met mutations in the ATP-binding site. Antibodies against CPLA2 and COX-2 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). [14C]Arachidonoylphosphatidylcholine was from Amersham Pharmacia Biotech.

The truncation mutant encoding the region from −102 to +12 (28) was used to generate additional truncations from phosphorylation reaction. The following primers were used: for the −58 mutant sense primer: GTGACTCGACAACGCTTCA; for the −37 mutant sense primer: GTGACTCGACAACGCTTCA; for the −12 mutant sense primer: GTGACTCGACAACGCTTCA. The antisense primer for all three was the same and was derived from the PA3-Luc vector: CCACACCCCTATGTAAACCAGTAGTACCC.

Cell Culture and Transfection—RL-65 lung epithelial cells were obtained from American Type Tissue Culture and grown in Dulbecco’s modified Eagle’s medium/F-12 supplemented with NaHCO3 (20 mM), sodium pyruvate (10 mM), insulin (5 μg/ml), transferrin (10 μg/ml), ethanolamine (100 μM), phosphoethanolamine (100 μM), hydrocortisone (0.5 μM), forskolin (5 μM), retinoic acid (50 nM), bovine pituitary extract (150 μg/ml), penicillin (100 units/ml), and streptomycin (100 units/ml), according to the producer’s recommendation. A549 human lung cancer cells were obtained from the University of Colorado Cancer Center Tissue Culture Core and grown in RPMI containing 10% fetal calf serum.

For transient transfections, cells were electroporated as described previously (2). Briefly, two million cells were electroporated in duplicate dishes using a geneZAPPER electroporator (IBI). Unless otherwise stated cells were transfected with 2 μg of the cPLA2 promoter, 2 μg of CMV-β-gal, and 2 μg of other DNA (e.g. H-Ras, BxB-Raf). Total DNA concentration for each transfection was matched with plasmid lacking an insert. Following electroporation, cells were incubated in standard media for 48 h. Cells were then harvested, and luciferase and β-galactosidase activity was determined as described previously (2). Results are expressed as luciferase units normalized to β-gal.

For stable transfection, H-Ras cDNA was inserted at the HindIII site of the retroviral expression vector pMVT (34) and packaged into a replication-deficient retrovirus as described previously (35). Polybrene (8 μg/ml) was added to the retroviral-containing medium from the 293T packaging cells and filtered prior to a 24-h incubation with subconfluent monolayers of RL-65 cells. The infected cells were replated, selected for G418 resistance, and expanded. Control cell lines (Neo) were selected by infecting cells with virus lacking a cDNA insert. Clones were selected by immunoblotting with anti-H-Ras antibodies.

The MAP kinase family: the stress-activated protein kinase/c-Jun amino-terminal kinases (JNKs) and the p38 family of MAP kinase (15, 16). The physiologic roles of JNKs and p38 MAP kinase appear to be complex and may depend on the context in which these enzymes are activated. Activation in response to cell stresses or growth factors is mediated through specific upstream kinases: MKK4/7 for JNKs (17, 18) and MKK3/6 for p38 MAP kinase (19, 20). Both JNKs and p38 MAP kinase have been shown to phosphorylate transcription factors, including c-Jun, ATF-2, and members of the ets family including Elk-1 (21).
To examine mechanisms leading to increased cPLA₂ expression, RL-65 cells were transiently cotransfected with a construct encoding 2.4 kb of the rat cPLA₂ promoter ligated to a promoterless luciferase reporter (PA3-Luc) along with an expression plasmid for H-Ras. Transient cotransfection of H-Ras increased cPLA₂ promoter activity 5- to 8-fold (Fig. 2A). Treatment of the cells with BZA-5B, a farnesyl transferase inhibitor, which has been shown to block Ras function (37), inhibited the induction of promoter activity, suggesting that this represents a direct effect of Ras. These data are consistent with our previous finding demonstrating inhibition of cPLA₂ promoter activity and protein expression in two NSCLC lines (2). Transient expression of H-Ras also increased COX-2 promoter activity (Fig. 2B), consistent with increased levels of expression of both enzymes detected in stable transfectants.

Ras activation results in activation of multiple MAP kinase pathways in other cell types. Activation of MAP kinase family members was determined by cotransferring RL-65 cells with plasmids encoding the activation domain of specific transcription factors fused to the DNA-binding domain of Gal4, along with a plasmid containing five Gal-4 binding domains upstream of a promoterless luciferase reporter (UAS-luc). Separate experiments were performed with Elk-Gal4, Jun-Gal4, and ATF-2-Gal4. Elk-Gal4-mediated increases in luciferase are a measure of ERK activity, because ERKs are the major kinase phosphorylating Elk. Jun-Gal4 activation is a measure of JNK activity, and ATF-2-Gal4 activation is a measure of JNK and p38 MAP kinase activity. Expression of H-Ras resulted in increased luciferase activity with all three Gal-4 fusions (Table I), indicating that H-Ras activates all three MAP kinase pathways in these cells. We therefore sought to examine the role of these pathways in regulation of the cPLA₂ promoter.

![Image](image-url)

**FIG. 1.** Induction of cPLA₂ and COX-2 expression in RL-65 cells by H-Ras. RL-65 cells were stably transfected with H-Ras or with empty vector (Neo), and stable transfectants were selected for G418 resistance. Results are shown for two representative clones (H-Ras-2 and H-Ras-31). A, cell lysates were prepared, and equal amounts of lystate protein were immunoblotted for cPLA₂ (top panel) or COX-2 (bottom panel). B, cell lysates from Neo and two clones were assayed for cPLA₂ activity using [14C]PC as substrate. Results represent the mean of triplicate determinations ± S.E. *, p < 0.01, **, p < 0.05 versus Neo.

**TABLE I**

| Construct       | UAS-alone | +Elk-Gal4 | +Jun-Gal4 | +ATF-2-Gal4 |
|-----------------|-----------|-----------|-----------|-------------|
| luciferase units/β-gal units |           |           |           |             |
| pcDNA-3         | 66 ± 1    | 405 ± 22  | 222 ± 37  | 656 ± 119   |
| H-Ras           | 405 ± 16  | 28456 ± 880° | 2111 ± 621° | 9601 ± 816° |
| BxB-Raf         | 76.2 ± 6.1 | 19950 ± 977° | ND°     | ND          |
| MEKK1           | 55 ± 1    | 6212 ± 226 | 2177 ± 135° | ND          |

*p < 0.05 versus UAS-luc alone.
° ND, not determined.

Activation of ERKs via Ras is mediated through activation of the serine-threonine kinase Raf. Expression of constitutively active Raf (BxB-Raf) increased ERK activity as assessed with Elk-Gal4 fusions to approximately the same extent as expression of H-Ras (Table I), but did not significantly increase cPLA₂ promoter activity (Fig. 2A) or COX-2 promoter activity (Fig.
2B). These results were confirmed by transient transfection of cells with epitope-tagged ERK-1, followed by immunoprecipitation and assay of kinase activity in the immunoprecipitate (data not shown). To further assess the role of the ERK pathway in regulation of the cPLA₂ promoter, transfected cells were treated with the MEK inhibitor PD98059 (38). Treatment with 50 μM PD98059, a concentration that blocks ERK activation in these cells, decreased the Ras-mediated induction of the cPLA₂ promoter by ∼50% (Fig. 3A). In separate studies, RL-65 cells were cotransfected with the cPLA₂ promoter construct and expression plasmids encoding H-Ras, with or without an expression plasmid encoding a kinase-dead form of ERK-1 (DN-ERK). Luciferase activity was determined as above. Results represent the mean of duplicate experiments with the S.E. indicated. *, p < 0.05 versus H-Ras alone.

It has recently been reported that chimeric proteins of JNK kinases have been constructed (32). We therefore examined expression plasmids encoding MKK7 fused to JNKs as activating JNKs (32). We therefore performed transient transfection experiments with a construct encoding a dominant negative form of ERK-1 or (DN-ERK). This construct has been used previously in studying the COX-2 promoter (39). Expression of constitutively active MEKK1 also increased cPLA₂ promoter activity (Fig. 4A). Because expression of wild-type MKK4 or MKK7 had no effect (data not shown). Correspondingly, expression of DN-MKK4 or DN-MKK7 also inhibited steady-state cPLA₂ promoter activity in A549 cells (Fig. 4B), a human NSCLC line expressing oncogenic forms of Ras (2). Therefore, we performed transient transfection experiments with the combination of enzymes above that obtained with the MKK7/JNK fusions increased promoter activity to ∼50% of the level achieved with expression of H-Ras. Combined, the data in Figs. 4 and 5 indicate that JNKs are involved in the induction of the cPLA₂ promoter. However, no further increase in promoter activity was found with the combination of enzymes above that obtained with the MKK7/JNK constructs alone (data not shown). To further test a cooperative role for JNKs and ERKs in driving the promoter, we performed transient transfection experiments with a constitutively active form of MEKK1. Expression of constitutively active MEKK1 also markedly increased cPLA₂ promoter activity (Fig. 6). Because expression of MEKK1 appeared to systematically increase ex-
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Fig. 5. Induction of cPLA₂ promoter by constitutively active JNK constructs. RL-65 cells were transiently transfected with the cPLA₂ promoter and plasmids encoding either H-Ras or fusion proteins of MKK7 linked to JNK3 (MKK/JNK3) or JNK2 (MKK/JNK2). After 48 h, lysates were prepared and assayed for luciferase and β-gal activity. The data shown represent the means of four independent experiments performed in duplicate with the S.E. indicated. **, p < 0.01 versus pcDNA-3 control.

Fig. 6. Induction of cPLA₂ promoter by constitutively active MEKK1. RL-65 cells were transfected with the cPLA₂ promoter plasmid and either 2 μg of constitutively active MEKK1, or pcDNA-3 as a control. All transfections also contained 2 μg of plasmid encoding Renilla luciferase under the control of a thymidine kinase promoter. After 48 h, cell lysates were prepared and assayed for both firefly luciferase and Renilla luciferase using the manufacturer’s protocol. Results represent the mean of two independent experiments performed in duplicate with the S.E. indicated. *, p < 0.05 versus pcDNA-3.

Fig. 7. Effect of I-κB-(Δ1–36) on induction of cPLA₂ promoter in lung epithelial cells. RL-65 cells were transfected without (solid bars) or with H-Ras (open bars) along with either the cPLA₂ promoter (A), COX-2 promoter (B), or κB-luc (C) as well as either pcDNA-3 or the dominant negative inhibitor of NF-κB inhibitor I-κB-(Δ1–36). After 48 h, cells were harvested and luciferase activity normalized to β-gal was determined. Results represent the mean of three independent experiments with the S.E. indicated. *, p < 0.05 versus pcDNA-3.

Expression of β-gal, presumably by activating the CMV promoter, we chose to use expression of Renilla luciferase driven by the thymidine kinase promoter to normalize for transfection efficiency in these experiments.

The role of p38 MAP kinase was examined by treating transfected cells with SB203580, a specific p38 MAP kinase inhibitor (40). At a concentration of 10 μM, which blocked H-Ras-mediated p38 MAP kinase activation (data not shown), no inhibition of the H-Ras-mediated induction of the cPLA₂ promoter was detected (Fig. 3A). Transient expression of constitutively active MKK6, which specifically activates p38 MAP kinase (20), also failed to increase promoter activity or affect the induction observed with expression of H-Ras (data not shown).

The cPLA₂ promoter contains a putative NF-κB-site at −760 to −751 (28), which has been suggested to be critical for induction of the enzyme. Transient expression of H-Ras in RL-65 cells increased NF-κB activity, as determined with a plasmid containing three consensus NF-κB sites (κB-luc). This induction was blocked by expression of a dominant negative form of I-κB lacking the regulatory phosphorylation sites required for dissociation from NF-κB (I-κB-(Δ1–36)) (Fig. 7A). However, expression of I-κB-(Δ1–36) failed to block the increase in cPLA₂ promoter activity observed with H-Ras (Fig. 7B), indicating that NF-κB does not contribute to Ras regulation of the cPLA₂ promoter. Induction of the COX-2 promoter was also not inhibited by expression of I-κB-(Δ1–36), and, in fact, induction of promoter activity by H-Ras was somewhat increased (Fig. 7C).

To begin to define the regions of the promoter required for induction by H-Ras, we prepared a series of truncations of the original 2.4-kb fragment (28). Removal of all but the last 58 bp of the promoter did not significantly change either basal promoter activity or H-Ras-mediated induction (Fig. 8A). Removal of an additional region down to −37 decreased basal and H-Ras-mediated induction but did not affect the -fold induction caused by Ras. Finally, truncation down to −12 abolished both basal and H-Ras-induced promoter activity. Parallel studies were also performed in A549 cells. Promoter activity slightly increased in going from −2.4 kb to −58 bp (Fig. 8B). A further decrease to −37 decreased promoter activity by ∼60%, and a further truncation to −12 abolished promoter activity completely. It thus appears that removing all but the last 58 bp of the promoter does not impair activity in either RL-65 cells or NSCLC, and the construct containing the region from −37 still retains some basal activity and can be induced by expression of H-Ras.
all three members of the MAP kinase family. By expression of Gal4 fusion proteins that encode for the activation domains of known substrates of these kinases, we were able to demonstrate activation of ERKs, JNKs, and p38 MAP kinase. These results were confirmed by direct measurements of kinase activities in cells stably expressing H-Ras (data not shown). Activation of the JNK pathway appears to be both necessary and sufficient for induction of cPLA2. Dominant negative forms of JNK kinases inhibited the Ras-mediated induction. Expression of these constructs also inhibited steady-state cPLA2 promoter activity in NSCLC, suggesting that similar signaling pathways mediate induction of cPLA2 in these cells. Expression of constitutively active JNK constructs, obtained by expressing fusion proteins of JNK fused to MKK7 increased cPLA2 promoter activity. A critical requirement for JNK activation in cPLA2 induction is consistent with a preferential role for this pathway in the mitogenesis of NSCLC (41).

Because activation of JNK activity resulted in only half of the induction seen with H-Ras, it is likely that additional pathways are required for full activation. The ERK pathway appears to be necessary but not sufficient to induce cPLA2 expression. Treatment of cells with a specific pharmacological MEK inhibitor, or expression of a dominant negative form of ERK, both decreased the Ras-mediated induction of the promoter by half, but expression of constitutively active Raf failed to significantly increase promoter activity and did not increase the induction seen with the constitutively active JNK constructs alone.

Expression of MEKK1, which like Ras, stimulated the activity of all the MAP kinase families, resulted in increases in promoter activity similar to that seen with H-Ras. These findings suggest that Ras, and probably MEKK1, engage additional signaling pathways critical for induction of the cPLA2 promoter. This finding was confirmed by the observation that, whereas inhibition of both the ERK and the JNK pathway resulted in further inhibition of promoter activity, residual induction of the promoter by Ras was still observed.

Activation of p38 MAP kinase does not appear to represent this additional pathway. Neither inhibition of p38 MAP kinase with a specific inhibitor nor expression of MKK6, which specifically activates p38 MAP kinase, had any effect on promoter activity. We have also observed that the p38 MAP kinase inhibitor did not alter expression of cPLA2 or COX-2 in several NSCLCs (data not shown). Further studies are required to identify these pathways.

A number of studies have examined induction of cPLA2 expression in nontransformed cells in response to circulating factors. The most potent agents mediating this induction appear to be cytokines. Interleukin-1β induced cPLA2 expression in human synovial fibroblasts (42). Tumor necrosis factor-α induced expression in Hep-2 cells (43), and HeLa cells (44), and interferon γ induced cPLA2 expression in BRAS-2B epithelial cells (45). A common effector pathway activated by these agents is NF-κB, and the observation that the cPLA2 contains a putative NF-κB caused us to examine this pathway. Although expression of H-Ras resulted in a marked activation of NF-κB, as assessed by a NF-κB-sensitive reporter, dominant negative IκB had no effect on induction of the cPLA2 promoter, and somewhat potentiated induction of the COX-2 promoter. It therefore appears that NF-κB is not involved in the Ras-mediated induction of these enzymes. Analogous studies are required to directly test whether this element of the promoter plays a role in cytokine-mediated induction. A number of studies have shown that expression of constitutively active forms of Ras leads to the production of specific cytokines. In fibroblasts, expression of H-Ras led to expression of granulocyte-colony

DISCUSSION

Expression of oncogenic forms of Ras is observed in a variety of cancer cells and is believed to play a critical role in the dysregulated growth of these cells. However, the downstream signaling pathways mediating these effects are less well defined. This is complicated by the identification of a large number of potential Ras effectors (see Ref. 12).

Previous studies from our laboratory have demonstrated that expression of oncogenic Ras in NSCLC is associated with increased expression of cPLA2 and COX-2, resulting in constitutively high levels of eicosanoid production in these cells. The ability of inhibitors of eicosanoid production to block anchorage-independent growth of NSCLC (2), as well as the growth of xenografts of NSCLC in athymic mice3 indicates that this pathway is critical for transformed growth.

Because NSCLCs are likely to manifest mutations in multiple signaling pathways, we sought to determine whether expression of constitutively active Ras is sufficient to induce enzymes in the eicosanoid pathway in normal lung epithelial cells. From our studies we conclude that expression of H-Ras is sufficient to increase expression of both cPLA2 and COX-2 in a nontransformed cell line. This induction is mediated through transcriptional activation of both promoters and is a direct effect of Ras.

As in other cell types, activation of Ras leads to activation of

3 L. E. Heasley, D. Chan, and R. A. Nemenoff, unpublished observations.

Fig. 8. Truncational mutants of the cPLA2 promoter. A, constructs encoding truncations of the rat cPLA2 promoter beginning at the indicated position were cotransfected into RL-65 cells along with either plasmid encoding H-Ras (+ H-Ras) or pcDNA-3 as a control (− H-Ras). Promoter activity normalized to β-gal activity was determined after 48 h in standard media. B, the same constructs were transfected into A549 cells, and promoter activity was determined after 48-h incubation. Results represent the mean of triplicate experiments with the S.E. indicated.
stimulating factor (CSF), granulocyte-macrophage-CSF, and interleukin-1β (46). Interestingly, elevated cytokine production has been demonstrated in a number of nonsmall cell lung cancer lines (47–49). However, the ability of H-Ras to induce the cPLA₂ promoter in transient transfection experiments, where only a small percentage of the cells are expressing the oncogene, makes it unlikely that the induction observed in RL-65 cells is mediated through cytokine production. Induction of COX-2 has been shown to be mediated by both NF-κB-dependent and independent pathways. Induction in response appears to require activation of NF-κB (50), whereas induction by platelet-derived growth factor or src signals through the JNK pathway (51, 52).

The lack of a role for NF-κB in regulating the cPLA₂ promoter is consistent with our truncational analysis, which demonstrated that removal of the NF-κB site did not affect inducibility of the promoter by H-Ras in RL-65 cells, or steady-state promoter activity in NSCLC. In fact, the minimal region of the promoter that we have defined from −58 to +12 does not contain any of the putative regulatory elements previously identified, suggesting that novel transcription factors may be involved. Induction of COX-2 through pathways involving H-Ras has been shown to also be mediated by JNKs as well as ERKs (53), and involves binding to a cAMP-response element in the COX-2 promoter (52). It thus appears that expression of H-Ras leads to induction of both COX-2 and cPLA₂ through similar downstream signaling pathways. However, these pathways diverge at the level of the specific transcription factors, and cis-acting regulatory factors regulating each promoter.

Increased expression of cPLA₂ has also been reported in Ras-transformed fibroblasts (54), resulting in constitutively elevated eicosanoid production, which presumably contributes to the transformed growth of those cells. Studies from our laboratory have demonstrated that, in vascular smooth muscle cells, activation of Ras also leads to increased cPLA₂ expression and eicosanoid production (35). In these cells increased cPLA₂ expression is not associated with increased proliferation but rather appears to be involved in suppression of muscle-specific gene expression, leading to a less differentiated phenotype.

In conclusion, induction of cPLA₂ represents a novel downstream effector of Ras, which is likely to play an important role in the physiologic responses following Ras activation in both normal and transformed cells.