Induction of cPLA$_2$ in Lung Epithelial Cells and Non-small Cell Lung Cancer Is Mediated by Sp1 and c-Jun*

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Activating mutations in ras genes are frequently associated with non-small cell lung cancer cells (NSCLC) and contribute to transformed growth in these cells. Expression of oncogenic forms of Ras in these cells is associated with increased expression and activity of cytosolic phospholipase A$_2$ (cPLA$_2$) and cyclooxygenase-2 (COX-2), leading to constitutively elevated levels of prostaglandin production. Expression of oncogenic Ras is sufficient to induce these enzymes in normal lung epithelial cells. We have previously reported that the JNK and ERK pathways are necessary for induction of cPLA$_2$ and have defined a minimal region of the cPLA$_2$ promoter from −58 to −12 that is required for Ha-Ras-mediated induction. To further characterize the cis-regulatory elements within this region involved in the response, site-directed mutagenesis was used to make mutations at various sites. Three cis-regulatory elements were identified: regions −21/−18, −37/−30, and −55/−53. Mutations in any of these elements decreased basal and Ha-Ras-induced cPLA$_2$ promoter activity in both normal lung epithelial cells, as well as steady state promoter activity in A549 cells, with a mutation in element −21/−18 completely eliminating all promoter activity. Overexpression studies and gel shift assays indicated that Sp1 may serve as a transcription factor functionally regulating promoter activity by directly interacting with two of the cis-regulatory elements, −21/−18 and −37/−30. Expression of Ha-Ras led to induction of c-Jun protein, which showed functional cooperation with Sp1 in driving promoter activity. Additional unidentified transcription factors bound to the regions from −55/−53 and −37/−34.

Lung cancer is the leading cause of cancer death in the United States, but, despite a significant research effort, the critical pathways mediating transformed growth remain poorly understood. Non-small cell lung cancer (NSCLC) constitutes the majority of lung cancers, and gain of function mutations in ras genes are frequently associated with NSCLC, occurring in 30% of adenocarcinomas, and just under 10% of other NSCLC types (1). Activating mutations in ras genes have also been detected in other types of human cancer, including colon, prostate, and pancreatic. It is believed that these mutated forms of Ras lacking 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 A$_2$ (cPLA$_2$) and cyclooxygenase-2 (COX-2) in a panel of NSCLC cell lines (2).

cPLA$_2$ is the major intracellular form of PLA$_2$, which preferentially hydrolyzes membrane phospholipids at the sn-2 position to release arachidonic acid and represents the rate-limiting enzyme in eicosanoid production (3–5). Free arachidonic acid is metabolized through three major pathways to produce eicosanoids. COX converts arachidonic acid to prostaglandins and thromboxane, lipoxigenases produce leukotrienes and hydroxyeicosatetraenoic acids, and cytochrome P-450 epoxygenase produces epoxyeicosatrienoic acids. Two forms of cyclooxygenase have been identified (6). COX-1 is constitutively expressed in most cell types and involved in maintaining vascular tone, whereas COX-2 is an immediate early response gene (7) induced by mitogenic stimuli and associated with inflammation. Constitutively high levels of prostaglandin production are observed in NSCLC as a result of elevated levels of cPLA$_2$ and COX-2 (8, 9). We (2) and others (10–12) have shown that nonsteroidal anti-inflammatory agents, which inhibit eicosanoid production, block the transformed growth of NSCLC expressing Ras mutations. These drugs do not hinder the growth of most nontransformed cells, suggesting that this pathway plays a critical role in transformation.

Although studies in NSCLC cell lines have implicated a role for Ras in the induction of cPLA$_2$, these cells contain a large number of mutations and aberrations in signaling pathways, making it difficult to define the critical molecular pathways regulating cPLA$_2$ expression. We therefore sought to examine the effects of constitutively active forms of Ras on cPLA$_2$ expression in untransformed lung epithelial cells. Ras has been shown to connect to numerous effector pathways, leading to the activation of diverse physiological responses (see Ref. 13 for review). These pathways regulate cell proliferation as well as changes in the cytoskeleton. We have recently demonstrated that expression of oncogenic forms of Ras directly increased cPLA$_2$ expression in normal lung epithelial cells through activation of the JNK and ERK pathways (14).

The regulatory elements within the cPLA$_2$ promoter critical for this induction by oncogenic Ras have not been defined. The cPLA$_2$ promoter has been isolated from both human (15) and rat (16) and contains a number of putative regulatory elements including AP-1 sites, NF-$κ$B sites, and glucocorticoid regulatory elements. The promoter region resembles that of a housekeeping gene in that it contains no TATA box and no CAAT box,

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§ The abbreviations used are: NSCLC, non-small cell lung cancer; cPLA$_2$, cytosolic phospholipase A$_2$; COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; bp, base pair, epidermal growth factor; CMV, cytomegalovirus; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; GKLF, gut Kruppel-like factor; LKLF, lung Kruppel-like factor; WT, wild type.
but it is atypical in that it is not GC-rich (34.5%) and has no consensus Sp1 sites (15, 17). We reported previously that the region of the promoter covering residues −58 to −12 is crucial for induction of promoter activity by oncogenic Ras (14) in RL-65 cells, a neonatal, untransformed rat epithelial cell line (18). In this study, we define three cis-regulatory elements within this region of the promoter critical for both basal and Ha-Ras-induced cPLA₂ promoter activity, and have begun to define transcription factors acting at these sites.

MATERIALS AND METHODS

Reagents and Constructs—The cPLA₂ promoter construct contains 2.4 kilobase pairs of the 5′ region ligated into the promoterless luciferase vector PA3-Luc (16). The truncation mutant encoding the region from −102 to +40 (16) was used to generate additional truncations by polymerase chain reaction as described previously (14). Mutations within this region were generated using the QuikChange site-directed mutagenesis kit (Stratagene). The following primers were used with mutations underlined in bold: mutation −55′−53 sense primer, CTGATCCCCGGGTACGTTTAAACATCCAGCAGAGCACG; mutation −55′−53 antisense primer, CGTGTCTCTGGTGGTGTAAAGGTTAGTACGTTGATCCAGAGCACG; mutation −48′−47 sense primer, CACCTTAACATCCACAGAGAGACCA; mutation −48′−47 antisense primer, CTGCTGTCTCTGTGTTACTTAAGGTTGGTTATCT. To define regulatory elements, a series of mutations were introduced throughout the cPLA₂ promoter spanning residues −102 to −58, which was necessary and sufficient for Ha-Ras-mediated induction of cPLA₂ in RL-65 cells and expression in NSCLC (14). An additional 5′ truncation down to −37 reduced basal promoter activity, and inhibited but did not completely block induction by Ha-Ras. Ultimate truncation down to −12 completely abolished both basal and Ha-Ras-induced promoter activity. These studies were performed in A549 cells, a human NSCLC cell line expressing an activating mutation in Ras. Promoter activity was slightly elevated upon truncation from −2.4 kilobase pairs to −58 bp. Further deletion to −37 decreased promoter activity by ~60%, and truncation to −12 entirely abolished promoter activity.

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Examination of the sequence of the cPLA₂ promoter revealed that the 58-bp region contained no consensus sites for any known transcription factors. Therefore, to define regulatory elements, a series of mutations were introduced throughout the region with the original −58 truncation mutant as a template (Fig. 1). With the exception of mutation −37′−34′, all mutations correspond to regions of the promoter that are conserved between the rat and human promoter (15). Plasmids encoding each mutation were transiently co-transfected into RL-65 cells along with an expression plasmid for Ha-Ras or pcDNA-3 as a control. Expression of Ha-Ras increased wild type promoter activity by 3–5-fold (Fig. 2A), consistent with earlier studies. Mutation −21′−18 almost completely abolished both basal and Ha-Ras stimulated promoter activity. Mutations −37′−34′ and −33′−30 inhibited both basal and Ha-Ras-mediated promoter activity by ~80–90%. With the double mutant −37′−30′, activity was further inhibited to ~10% of wild-type promoter. Mutation at −55′−53 decreased both basal promoter activity and Ha-Ras-mediated induction by 66%. There was no significant change in either basal promoter activity or Ha-Ras-mediated induction with mutations −49′−47.

Stimulation of RL-65 cells with EGF also increased cPLA₂ promoter activity (Fig. 2B). This induction was blocked by the combination with M17-Ras (data not shown), indicating that the effects of EGF are mediated at least in part through the Ras signaling pathway. EGF stimulation was blocked with mutants −55′−53, −37′−34, −33′−30, or −21′−18 to an extent similar to that seen with expression of Ha-Ras (Fig. 2B). Finally, the effects of these mutations in the cPLA₂ promoter were also examined in A549 cells. Steady-state promoter activity was
almost entirely eliminated with mutation -21/-18. Mutations -55/-53, -37/-34, and -33/-30 inhibited steady-state promoter activity by about 95%. Mutation -49/-47 reduced promoter activity by 60%.

The data from these studies suggest that three regions of the cPLA₂ promoter appear to be critical for expression: regions -37/-30, -21/-18, and -55/-53. To examine complexes formed at these sites, EMSAs were performed using 32P-labeled probes spanning each of the three cis-regulatory elements (Figs. 4–6).

Using an oligonucleotide encoding -42 to -13, a number of specific bands were detected using nuclear extracts prepared from RL-65 cells (Fig. 4A). At least three complexes were competed off by excess cold oligonucleotide. Extracts from EGF-stimulated cells showed the same pattern of bands, with no significant change in intensity (data not shown). Sp1 is a ubiquitously expressed transcription factor, which binds to GC-rich elements frequently found in a number of housekeeping genes. The region of the cPLA₂ promoter from -58 to -1 is 55% GC-rich, and several areas within this region represent putative, but not consensus Sp1 binding sites. Sp1 has been demonstrated to activate numerous TATA-less promoters (20, 21) and has recently been shown to control expression of tissue specific genes as well (22, 23). To examine whether Sp1 can form complexes with this region of the cPLA₂ promoter, we employed a specific Sp1 antibody in a "supershift" EMSA. In the presence of Sp1 antibody and nuclear extracts from RL-65

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**Table 1.** Site-directed mutagenesis of the -58/+40 region of the rat cPLA₂ promoter. QuikChange site-directed mutagenesis was used to construct plasmids containing the 58-bp region of the rat cPLA₂ promoter with the shown mutations inserted into a promoterless pA3-LUC vector. The various mutations are **bold** and **underlined**. The probes used in electrophoretic mobility shift assays are **underlined** in the wild type sequence.

| Mut   | WT Sequence | Mutated Sequence |
|-------|-------------|-----------------|
| -55/-53 | TCC ACC TTA ACA TCC ACA GAG ACC AGC CCA TTT CTT AGC CCC TCC TAC CAG CGG GAG A | TCC GTT TTA ACA TCC ACA GAG ACC AGC CCA TTT CTT AGC CCC TCC TAC CAG CGG GAG A |
| -49/-47 | TCC ACC TTA AGT ACC ACA GAG ACC AGC CCA TTT CTT AGC CCC TCC TAC CAG CGG GAG A | TCC ACC TTA ACA TCC ACA GAG AGT ACC ACA GAG ACC AGC CCA TTT CTT AGC CCC TCC TAC CAG CGG GAG A |
| -37/-34 | TCC ACC TTA ACA TCC ACA GGT AGT ACC ACA GAG ACC AGC CCA TTT CTT AGC CCC TCC TAC CAG CGG GAG A | TCC ACC TTA ACA TCC ACA GAG ACC AGT ACA TTA TGA TTT CTT AGC CCC TCC TAC CAG CGG GAG A |
| -33/-30 | TCC ACC TTA ACA TCC ACA GAG ACC ATA TGA TTT CTT AGC CCC TCC TAC CAG CGG GAG A | TCC ACC TTA ACA TCC ACA GAG GGT ATA TGA TTT CTT AGC CCC TCC TAC CAG CGG GAG A |
| -21/-18 | TCC ACC TTA ACA TCC ACA GAG ACC AGC CCA TTT CTT ATA TTC TCC TAC CAG CGG GAG A |
cells, a major complex (a) was supershifted to a slower migrating complex (b) (Fig. 4A). Using a probe with mutations at −33/−30, the intensity of band a was greatly diminished, and a new band (c) was detected (Fig. 4B). This band was not supershifted with anti-Sp1 antibody. Using a probe with mutations at −37/−34, resulted in decreased intensity of band a, and a detectable, but faint shifted band b (Fig. 4C). The double mutation −37/−30 eliminated all three bands (Fig. 4D).

A similar pattern of bands was observed using nuclear extracts prepared from A549 cells (Fig. 4E), with the slowest migrating band supershifted with anti-Sp1 antibodies. Mutations at −33/−30 caused a similar disappearance of band a and appearance of a new band (c).

With a labeled probe encoding the region from −28 to −8, three major bands were detected using extracts from either RL-65 cells or A549 cells (Fig. 5). All of these bands were competed off by excess cold oligonucleotide (data not shown). The slowest migrating band (a) was supershifted with anti-Sp1 anti-
bodies to a slower migrating complex (b). When the identical extracts were incubated with probe containing a mutation at −21/−18, no specific bands were detected with extracts from RL-65 cells (Fig. 5A, lane 1) or A549 cells (data not shown). Finally, EMSA analysis was performed using a labeled probe corresponding to the region from −28 to −8 of the cPLA2 promoter (lanes 1–4) or with probe containing mutations at −21/−18 (lane 5). Extracts were incubated with Sp1 antibody, c-Jun antibody, or no addition as described in Fig. 4. A specific complex (a) was supershifted to a slower migrating form (b) in the presence of Sp1 antibody. Right panel, extracts from A549 cells were incubated with WT probe in the presence or absence of Sp1 or c-Jun antibody.

FIG. 5. EMSA analysis in region −28/−8 of the cPLA2 promoter. Left panel, extracts from RL-65 cells were incubated with 32P-labeled probe corresponding to region−28 to −8 of the cPLA2 promoter (lanes 1–4) or with probe containing mutations at −21/−18 (lane 5). Extracts were incubated with Sp1 antibody, c-Jun antibody, or no addition as described in Fig. 4. A specific complex (a) was supershifted to a slower migrating form (b) in the presence of Sp1 antibody.

DISCUSSION

Activating mutations in Ras are characteristic of a variety of cancer cells and play a critical role in the transformation of these cells. We have shown previously that expression of oncogenic Ras is necessary for elevated expression of cPLA2 and COX-2 in NSCLC (2), and sufficient to induce expression of both enzymes in nontransformed lung epithelial cells (14). In both cell types, induction of cPLA2 involved activation of the ERK and the JNK mitogen-activated protein kinase pathways and in nontransformed lung epithelial cells (14). In both cell types, induction of cPLA2 involved activation of the ERK and the JNK mitogen-activated protein kinase pathways and in nontransformed lung epithelial cells (14). In both cell types, induction of cPLA2 involved activation of the ERK and the JNK mitogen-activated protein kinase pathways and in nontransformed lung epithelial cells (14). In both cell types, induction of cPLA2 involved activation of the ERK and the JNK mitogen-activated protein kinase pathways and in nontransformed lung epithelial cells (14). In both cell types, induction of cPLA2 involved activation of the ERK and the JNK mitogen-activated protein kinase pathways and in nontransformed lung epithelial cells (14).

Through truncational analysis, we have previously defined the minimal region of the promoter required for induction of cPLA2 from −58 to −12 (14). Further truncation of this region indicated two smaller regions, which contribute to regulated expression. Promoter activity decreased when truncating from −58 to −37, but some basal activity was retained and the -fold induction of the promoter achieved by expression of Ha-Ras was similar. An additional truncation to −12 abolished both basal and Ha-Ras stimulated activity. We sought to further characterize the cis-
regulatory elements in this region responsible for these effects. From the present study, we have identified three cis-regulatory elements from −55/−53, from −37/−30, and from −21/−18, which appear to be critical for regulated expression of cPLA2. In normal lung epithelial cells, mutations in each of these regions significantly decreased both basal promoter activity and stimulated promoter activity in response to expression of Ha-Ras or exposure to EGF. Similarly, each mutation decreased steady-state promoter activity in an NSCLC line expressing oncogenic Ras (A549). Overall, region −21/−18 appears to be the most crucial element in regulating promoter activity because mutation of this region abolishes all promoter activity.

By EMSA analysis, Sp1 bound to both the −37/−30 region and the −21/−18 region. Although these regions do not contain classical Sp1 sites, they are GC-rich. Our data suggest that at least two factors bind to the −37/−30 region. Mutating the −37/−34 region decreased the binding of Sp1, but still resulted in a fainter band, which was shifted by anti-Sp1 antibody (Fig. 4C). However, mutation of the −33/−30 region resulted in the formation of a complex that was not supershifted (Fig. 4B). We would therefore propose that a factor binding to the −37/−34 cooperates to promote Sp1 binding to the −33/−30 region. The identity of this second factor is unknown, but, based on supershift assays, it is not likely to be another member of the Sp1 family. Sp1 binding correlated with the ability of Ha-Ras to induce the promoter, because mutations that inhibited induction of promoter activity blunted or abolished Sp1 binding. Furthermore, overexpression of Sp1 in RL-65 cells was sufficient to induce promoter activity in the absence of Ha-Ras expression. Overexpressing Sp1 alone did not stimulate promoter activity to the extent seen with Ha-Ras. This may be a result of the fact that Sp1 is already highly expressed in most cells and is difficult to overexpress. Alternatively, Sp1 itself may not be sufficient to drive the promoter and an additional factor(s) is required (see above). Mutations in the −33/−30 region, which resulted in disappearance of complexes contain-
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...critical for activation of c-Jun-mediated expression through three cis-regulatory elements within its promoter, from −21 to −18, −37 to −30, and −55 to −53. A candidate transcription factor includes Sp1 which may bind to both of the elements, −21 to −18 and −33 to −30. Additional factors bind to the region from −37 to −34 and the third element from −55 to −53. c-Jun expression is induced by Ha-Ras and may play a functional role by interacting with Sp1 without directly binding to the promoter. The similarities in the pathways mediating induction of cPLA₂ and 12-lipoxygenase by Ha-Ras also suggest that the coordinated induction of these enzymes is required for enhanced eicosanoid production.

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