

Lung Krüppel-like factor (LKLF) is a transcriptional activator of the cytosolic phospholipase A₂ α promoter

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Increased expression of cPLA₂ (cytosolic phospholipase A₂) has been shown to be the cause of tumorigenesis of NSCLC (non-small-cell lung cancer). Our laboratory has previously demonstrated that oncogenic forms of Ras increase transcription of cPLA₂ in normal lung epithelial cells and NSCLC lines through activation of the ERK (extracellular-signal-regulated kinase) and JNK (c-Jun N-terminal kinase) MAPK (mitogen-activated protein kinase) family. We have also defined a minimal region of the cPLA₂ promoter that is critical for this induction. To identify potential transcription factors that bind to this region and regulate expression, a yeast one-hybrid screen was performed with a rat lung cDNA library. Multiple members of the Krüppel family were identified, with LKLF (lung Krüppel-like factor) being isolated a number of times. Overexpression of LKLF in lung epithelial cells or *Drosophila* SL-2 cells increased cPLA₂ promoter activity. Conversely, expression of a dominant negative form of LKLF inhibited induction of cPLA₂ promoter activity by

oncogenic Ras in normal lung epithelial cells and NSCLC. By electrophoretic mobility-shift assay analysis, it was found that LKLF bound to a GC-rich region of the cPLA₂ promoter located between –37 and –30 upstream from the transcription start site. Expression of siRNA (small interfering RNA) directed against LKLF inhibited basal expression of cPLA₂ in lung epithelial cells and blocked induction by H-Ras. In NSCLC, siRNA against LKLF co-operated with siRNA against Sp1 (stimulatory protein 1) to inhibit cPLA₂ promoter activity. Finally, recombinant LKLF was a substrate for ERKs. These results indicate that LKLF is an important regulator of cPLA₂ expression and participates in the induction of this protein, which is critical for increased eicosanoid production associated with lung tumorigenesis.

Key words: cytosolic phospholipase A₂ (cPLA₂), gene transcription, lung Krüppel-like factor (LKLF), non-small-cell lung cancer (NSCLC), small interfering RNA (siRNA), yeast one-hybrid.

INTRODUCTION

Eicosanoids are a family of lipid mediators derived from metabolism of arachidonic acid, which have been implicated in the regulation of cell growth, gene expression and inflammation [1]. There is a growing body of evidence indicating that eicosanoids, particularly prostaglandins, are involved in the etiologies of cancer (see [2–6] for a review). Increased levels of eicosanoids occur in a number of different types of human cancer, including colon, pancreatic, breast and lung. In the case of lung cancer, which is the leading cause of cancer death in the United States [7], increased prostaglandin biosynthesis has been found to occur mainly in NSCLC (non-small-cell lung cancer, which comprises 80% of lung cancers) rather than SCLC [8–10]. Inhibitors of prostaglandin production block the growth of NSCLC cell lines [11,12], as well as lung tumours in mice [13].

The rate-limiting step in the biosynthetic pathway of eicosanoids is the release of free arachidonic acid from the *sn*-2 position of membrane phospholipids by PLA₂ (phospholipase A₂). Among the PLA₂s, the physiologically most important form in this regard is the family of cPLA₂ (cytosolic PLA₂), primarily cPLA₂- α , which we will refer to, for brevity, as cPLA₂ in this paper [14–17]. Acutely, cPLA₂ activity is regulated by intracellular Ca²⁺ and phosphorylation. Increases in Ca²⁺ result in translocation to the nuclear envelope and activation. Phosphorylation, through MAP kinase (mitogen-activated protein kinase) pathways, also contributes to activation [18]. In addition to acute regulation, expression of cPLA₂ through changes in gene transcription is

mediated by a number of agents including cytokines and growth factors [19,20]. Our laboratory has demonstrated that in NSCLC, expression of oncogenic forms of Ras leads to induction of cPLA₂ through increased transcription and activation of the cPLA₂ promoter [12,21,22]. The promoter for cPLA₂ has been isolated from both human [20,23,24] and rat [25] genomes. A number of putative binding sites for possible regulatory elements have been identified within the promoter, including AP-1 (activating protein 1) sites, nuclear factor κ B sites and glucocorticoid regulatory elements [19]. In rat lung epithelial cells, induction of the cPLA₂ promoter by constitutively active Ras is mediated through the JNK (c-Jun N-terminal kinase) and ERK (extracellular-signal-regulated kinase) MAP kinase pathways [21]. Truncation of a 2.4 kb region of the promoter fragment down to the last 58 bp of the 5' untranslated region did not significantly change either basal promoter activity or H-Ras-mediated induction of the promoter [21]. Further mutagenesis studies within this region identified three regulatory regions and indicated that the transcription factor Sp1 (stimulatory protein 1) can bind to two of these regions [22].

Sp1 is a member of a family of transcription factors called SP/XKLF [26,27]. Members of this family contain three C2H2-type zinc fingers comprising the DNA-binding domain that is found close to the C-terminus. The KLFs (Krüppel-like factors), which form a subset of this family, are mammalian transcription factors with high similarity to the *Drosophila melanogaster* regulator protein Krüppel. Currently, at least 16 mammalian KLFs have been identified, with many more estimated to be present in the mammalian genome. All of these factors are surmised to bind to and

Abbreviations used: cPLA₂, cytosolic phospholipase A₂; DN-LKLF, dominant-negative LKLF; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; KLF, Krüppel-like factor; LKLF, lung KLF; MAP kinase, mitogen-activated protein kinase; NSCLC, non-small-cell lung cancer; siRNA, small interfering RNA; Sp1, stimulatory protein 1.

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interact with GC-rich DNA elements in promoters, enhancers and locus control regions [26,28,29].

In the present study, we sought to identify transcription factors binding to the proximal region of the cPLA₂ promoter, using a yeast one-hybrid approach. We report the identification of LKLF (lung KLF; KLF-2) as a transcriptional activator of cPLA₂, which binds to the proximal region of the cPLA₂ promoter and may interact with other members of the Sp1 family, and present evidence that LKLF participates in regulated expression of cPLA₂.

EXPERIMENTAL

Yeast one-hybrid

Transcription factors binding to a part of the rat cPLA₂ promoter were cloned using a yeast one-hybrid system (MATCH-MAKER One-Hybrid System; BD Biosciences Clontech, Palo Alto, CA, U.S.A.). Sense and antisense strands of three tandem repeats of a portion (−40 to −11; GAGACCAGCCCCATTCT-TAGCCCCCTCCTAC; 'target') of the rat cPLA₂ promoter were synthesized, annealed and cloned into the pHisi-1 and pLacZi vectors; yeast strain YM 4271 was transformed with the target oligo in each vector. YM 4271 with integrated target-pLacZi was doubly integrated with target-pHisi-1 and used as a parent cell for library screening. A rat lung cDNA library in the pACT2 vector (containing the GAL4 activation domain) was obtained from Clontech (catalogue no. RL400 6AH) and amplified. Screening of this rat lung cDNA library (50 µg of cDNA) was performed three times, by introducing the cDNA into the doubly integrated yeast strain and selecting for growth on SD/-His/-Leu plates (where SD stands for synthetic dropout media) containing 50 mM 3-AT (3-amino-1,2,4-triazole). After incubation of the plates at 30 °C for 4 days, several larger colonies were picked up and regrown on duplicate 100 mm plates to test for false positive clones by β-galactosidase filter assay. cDNA from the remaining positive yeast clones was 'rescued' by direct transfer of plasmid DNA from yeast to *Escherichia coli* by electroporation as described by Marciel and Higgins [30]. Miniprep DNA was prepared from the *E. coli* and the sequences of the cDNA compared with those in GenBank® database by the BLAST algorithm.

Expression plasmids and siRNA (small interfering RNA) constructs

An expression plasmid for full-length LKLF in the pBK-CMV vector was kindly provided by Dr J. Lingrel (University of Cincinnati, Cincinnati, OH, U.S.A.). The cDNA was cloned into the *Bam*HI and *Xba*I sites of the pcDNA 3.1+ vector (Invitrogen, Carlsbad, CA, U.S.A.) for transient expression in RL-65 cells. For expression in *Drosophila* SL-2 cells, the appropriate cDNA was cloned into the *Bam*HI and *Kpn*I sites of the pACT vector [31]. For *in vitro* expression as a GST (glutathione S-transferase)-fusion protein, the cDNA was cloned into the *Sal*II and *Not*I sites of the pGEX-5X-3 vector (Amersham, Piscataway, NJ, U.S.A.). The fusion proteins were subsequently purified by passage over glutathione-agarose (G4510; Sigma, St. Louis, MO, U.S.A.) before use in EMSAs (electrophoretic mobility-shift assays). DN-LKLF (dominant-negative LKLF), which contains only the DNA binding domain of LKLF, was a gift from Dr R. S. Kawahara (Department of Pharmacology, University of Nebraska Medical Center). Plasmids encoding siRNAs for LKLF and Sp1 were obtained from GenScript Corporation (Edison, NJ, U.S.A.). The specific sequence for the siRNA against rat or human LKLF (AT-GACGACCTCAACAACGTTTCAAGAGAACGTTGTTGAG-GTCGTCAT) was designed using the GenScript software, and ligated into the pU6 vector between the *Bam*HI and the *Hind*III site. This encodes a hairpin structure that will be processed to

produce the mature siRNA. Similarly, the sequence for the siRNA directed against rat or human Sp1 was TGGCTGGCAGATCA-TCTCTTTCAAGAGAAGAGATGATCTGCCAGCCA.

Western blotting and measurement of cPLA₂ activity

Cell collection, electrophoresis and immunoblotting were performed as described in [32]. Antibody against cPLA₂ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-LKLF antibodies were a gift from Dr J. Leiden (Abbott Pharmaceuticals, Abbott Park, IL, U.S.A.) and Dr L. Glimcher (Harvard School of Public Health), or were purchased from Santa Cruz Biotechnology. For measurements of cPLA₂ activity, cells were lysed in a homogenization buffer containing 50 mM Hepes (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors using a Dounce homogenizer. Activity in a high-speed supernatant was determined using [¹⁴C]arachidonoyl phosphatidylcholine in the presence of 5 mM Ca²⁺ as described previously [16].

Cell culture and transfections

RL-65 lung epithelial cells were grown and transiently transfected by electroporation as described previously [21]. Non-small-cell human lung cancer cell lines (H-2122) were obtained from the University of Colorado Health Sciences Center Tissue Culture Core and grown in RPMI containing 10% (v/v) fetal bovine serum in a humidified 5% CO₂ atmosphere. SL2 cells (Schneider's *Drosophila* line 2 cells) were obtained from A.T.C.C. (CRL-1963; Manassas, VA, U.S.A.) and grown in a loosely attached monolayer in Schneider's insect media (Invitrogen or Sigma) plus 10% heat-inactivated fetal bovine serum at 23 °C. For transient transfections, RL-65 cells were plated (500 000 cells/60 mm dish) and transfected on the following day using Lipofectamine™ (Invitrogen) according to the manufacturer's instructions with 0.5 µg of each plasmid. After 48 h, cells were harvested and luciferase activity normalized to β-galactosidase was determined.

For SL-2 cells, 1.5 × 10⁶ cells were plated in 60 mm dishes, and transfected the following day with plasmid cDNA using Lipofectin® reagent (Invitrogen) according to the manufacturer's instructions in serum-free media (1 µg of cPLA₂ promoter construct linked to luciferase and 0.1 µg of transcription factor construct in pACT, per transfection). After a 24 h incubation, the medium was changed to full culture medium and the cells were incubated for an additional 48 h. Cells were rinsed three times in PBS and lysed in reporter lysis buffer (Promega). Luciferase activity was measured using the luciferase assay kit from Promega. Protein concentrations were determined by the use of the Bradford assay from Bio-Rad (Hercules, CA, U.S.A.). All activities were normalized to the amount of protein present in each sample. To obtain RL-65 cells stably expressing siRNA against LKLF, cells were transfected with Lipofectamine™, and G418-resistant clones were selected. Clones were screened for expression by immunoblotting with anti-LKLF antibody. Two independent clones, which showed no detectable expression of LKLF were selected for further characterization.

EMSA

Cell nuclear extracts were prepared, and EMSAs and supershift assays with antibodies were performed essentially as described in [22]. Double-stranded oligonucleotides for gel-shift assays were fill-in labelled with [³²P]dCTP as described in [22]. The following oligonucleotides representing the −58/−12 region of the cPLA₂ promoter were used, with the mutations underlined in boldface:

wild-type, TCCACCTTAACATCCACAGAGACCAGCCATT-TCTTAGCCCCCTCCT; F-mut (-21/-18), TCCACCTTAACA-TCCACAGAGACCAGCCATTCTTAATTCTCCT; DE mut (-37/-30), TCCACCTTAACATCCACAGAGGTTATATGATTTCTTAGCCCCCTCCT. For competition assays, 50–100-fold excess of unlabelled oligonucleotides were used. Supershift assays were performed using a specific LKLF antiserum; control samples were incubated with the same concentration of normal rabbit serum. Nuclear extracts or fusion proteins (2 µg) were incubated in binding reaction buffer [60 mM KCl, 1 mM zinc acetate, 5 mM MgCl₂ and 0.5 µg of poly(dI-dC)] along with the relevant antibodies for 30 min on ice. [³²P]dCTP-labelled probes (100 000 c.p.m., 5 nmol) were added for an additional 30 min in a total volume of 30 µl. Samples were resolved on a non-denaturing 5% (w/v) acrylamide gel (29:1, acrylamide/bisacrylamide) in 1 × TGE (25 mM Tris, 1.0 mM EDTA and 190 mM glycine) at 25 mA/gel for approx. 180 min. Gels were dried and exposed to a film for autoradiography.

In vitro phosphorylation

For activation of ERK, L-65 cells were stimulated for 5 min with 10⁻⁸ MEGF (epidermal growth factor). Cells were rinsed twice in PBS and lysed in MAP kinase lysis buffer (0.5% Triton X-100, 50 mM β-glycerophosphate, pH 7.2, 0.1 mM sodium vanadate, 2 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 2 µg/ml leupeptin and 4 µg/ml aprotinin). Extracts were centrifuged for 5 min at 10 000 g and supernatants matched for protein. Extracts (0.5 mg of protein in 0.5 ml) were incubated for 1 h at 4°C with 1 µg of anti-ERK-1 and 1 µg of anti-ERK-2, and then for an additional 1 h with 100 µl of a 10% suspension of formalin-fixed *Staphylococcus aureus* bacteria (Sigma). Immune complexes were collected by centrifugation, washed three times with lysis buffer and resuspended in 75 µl of lysis buffer. Kinase activity in the immunocomplex was determined using either myelin basic protein as described previously in [33] or GST-LKLF. Samples were analysed by SDS/PAGE and autoradiography.

RESULTS

Yeast one-hybrid screen

We have previously reported that the region of the cPLA₂ promoter from -58 to +12 was sufficient to mediate induction by H-Ras in lung epithelial cells [21,22]. To identify transcription factors that bind to cPLA₂ promoter and regulate its expression, a yeast one-hybrid screening was performed using an oligonucleotide containing three tandem repeats of the region from -40 to -11 of the promoter as bait. With a yeast strain doubly integrated with this target-reporter construct in two different plasmids, a rat lung cDNA library in the pACT2 vector was screened three times. Out of a total of 21 positive yeast clones, eight clones had a DNA sequence corresponding to portions of the rat LKLF [34] (Table 1). These clones were of various lengths, but all were missing part of the N-terminal end. The longest clone contained approx. 74% of the protein coding region of LKLF. In addition to LKLF, a number of clones (out of the 21 positive clones) were isolated once. These are listed in Table 1. Most of these correspond to transcription factors that bind DNA through 3–6 zinc-finger sequences.

Overexpression of LKLF increases cPLA₂ promoter activity

Because LKLF was identified many times in the one-hybrid screen, we focused our efforts on confirming and studying the effects that LKLF may have on cPLA₂ transcription. By immunoblotting, LKLF is expressed in the lung epithelial cell line RL-65

Table 1 Clones isolated from a rat lung cDNA library

Clones were isolated from the rat lung cDNA library as described in the Experimental section. After sequencing, 21 clones were identified using the BLAST algorithm.

Clones isolated many times:
LKLF – isolated eight times
Clones isolated once:
Other Krüppel-like Zn finger cDNAs
Zf9
GKLF
Kidney or cardiovascular system-enriched KLF
Other Zn finger cDNAs
Sp3 (three Zn fingers)
Pur-1 (homologous with MAZ or c-Myc-associated Zn finger; six Zn fingers)
Vascular endothelium Zn-finger protein (Vezf1, DB1; six Zn fingers)
Other cDNAs:
Surfactant protein B

(see Figure 4), where we have demonstrated Ras induction of cPLA₂ expression [21], as well as in NSCLC lines (results not shown). To assess the role of LKLF on cPLA₂ expression, we examined the effects of overexpression of LKLF on cPLA₂ promoter activity and protein expression. RL-65 cells were co-transfected with plasmids encoding full-length LKLF along with either the full-length cPLA₂ promoter or a minimal region of the promoter spanning from -58 to +12 [22]. With the minimal promoter constructs, expression of LKLF increased the promoter activity by approx. 2-fold (Figure 1A), suggesting that LKLF is an activator of cPLA₂ expression. Similar induction was seen with the full-length promoter (results not shown). Expression of H-Ras increased promoter activity by 5–8-fold as shown previously [21], and overexpression of LKLF did not significantly affect Ras induction. To implicate a role for LKLF in Ras-mediated induction of the cPLA₂ promoter, cells were co-transfected with a truncated mutant of LKLF that expresses the DNA binding domain, but does not contain the activation domain (DN-LKLF). Expression of DN-LKLF blocked the ability of Ras to induce cPLA₂ expression by approx. 50% (Figure 1A). We have previously demonstrated that c-Jun and members of the Sp1 family co-operate to induce cPLA₂ promoter activity [22]. To examine potential interactions between c-Jun and LKLF, RL-65 cells were co-transfected with expression plasmids for c-Jun and LKLF. Expression of c-Jun further stimulated LKLF-dependent promoter activity resulting in an additive stimulation, similar to what has been seen with Sp1 (Figure 1B). To assess the role of LKLF in regulating expression of cPLA₂ in NSCLC, we determined the effects of overexpressing LKLF or DN-LKLF on cPLA₂ promoter activity in H2122 cells. These cells express gain-of-function Ras, which leads to high levels of cPLA₂ and cyclo-oxygenase-2 expression [12]. Overexpression of wild-type LKLF did not significantly alter promoter activity; however, expression of DN-LKLF markedly decreased steady-state promoter activity in these cells (Figure 1C).

Expression of LKLF in *Drosophila* SL-2 cells

Since Krüppel family members are expressed in mammalian cells, including RL-65 cells, we also examined the role of LKLF in regulating cPLA₂ promoter activity in *Drosophila* SL-2 cells, which do not express Krüppel family members [35]. Full-length LKLF, Sp1 and Sp3 were ligated into the pACT *Drosophila* expression vector. Transfection of SL-2 cells with full-length LKLF increased cPLA₂ promoter activity by approx. 10-fold (Figure 2A). Transfection of Sp3 gave a similar induction, whereas expression of Sp1 resulted in a 3–5-fold increase in promoter activity. Interestingly, expression of LKLF along with either Sp1

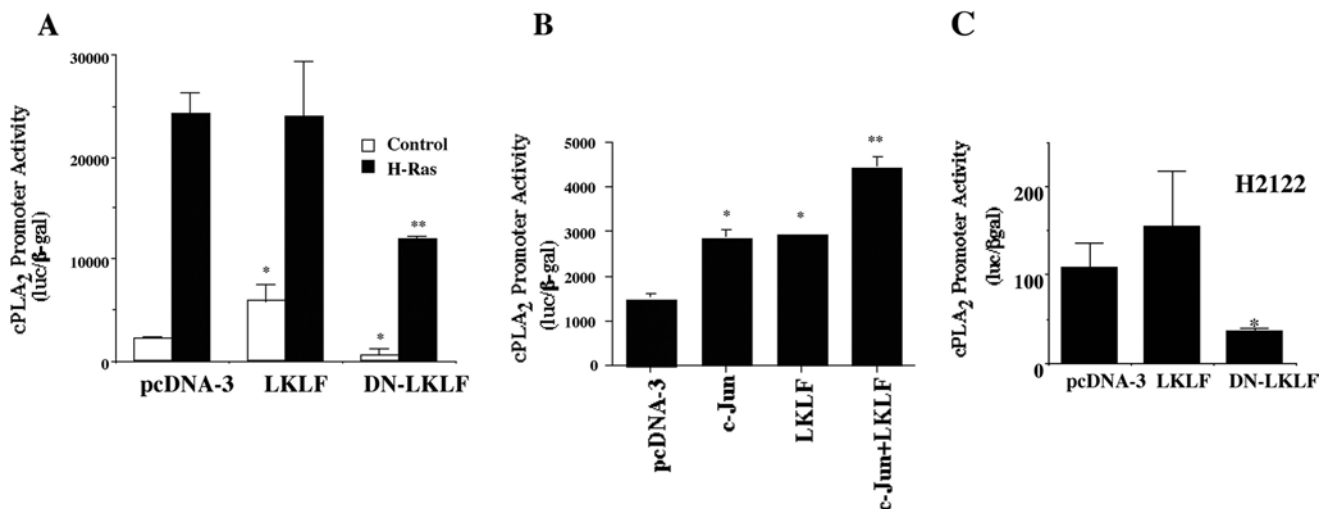


Figure 1 Expression of LKLF regulates cPLA₂ promoter activity in RL-65 cells

(A) RL-65 cells were transfected using Lipofectamine™ with the indicated plasmids along with a plasmid encoding the region of the cPLA₂ promoter from –58 to +12 driving luciferase and a plasmid encoding β-galactosidase to normalize for transfection efficiency. Cells were harvested 48 h after transfection, and luciferase normalized to β-gal (β-galactosidase) was determined. Expression of LKLF increased basal cPLA₂ promoter activity, and dominant negative LKLF decreased both basal and H-Ras-induced promoter activity. **P* < 0.05 versus basal with pcDNA-3; ***P* < 0.05 versus H-Ras alone. (B) RL-65 cells were transfected with expression plasmids encoding c-Jun, LKLF, or both together, along with the cPLA₂ promoter. Cells were harvested after 48 h and normalized promoter activity was determined. **P* < 0.05 versus pcDNA-3; ***P* < 0.05 versus either c-Jun or LKLF alone. (C) H2122 cells (an NSCLC cell line) were transfected with expression plasmids encoding LKLF or DN-LKLF along with the cPLA₂ promoter construct as in (A). Cells were harvested after 48 h. **P* < 0.05 versus pcDNA-3.

or Sp3 resulted in a synergistic increase in promoter activity. This was not observed with co-expression of Sp1 and Sp3, which gave an additive increase in promoter activity. To define regions of the promoter required for induction by individual Krüppel family members, we used a series of constructs encoding mutations in the minimal region of the cPLA₂ promoter, which we have previously used in RL-65 cells [22]. Mutations at positions –21/–18 completely abolished increase in promoter activity seen with LKLF, Sp1 or Sp3 (Figure 2B). Mutations in the –37/–30 region resulted in a 50% decrease in promoter activity in response to overexpression of each of the factors (Figure 2B). However, synergy between LKLF and either Sp1 or Sp3 was still observed.

EMSA analysis

EMSA using nuclear extracts from RL-65 cells result in a complex pattern of bands [22]. To determine whether LKLF forms a complex with the minimal region of the cPLA₂ promoter, we prepared a GST-fusion protein encoding full-length LKLF coupled with GST. This construct was expressed in *E. coli* and purified using glutathione-agarose. Purified GST–LKLF was incubated with labelled probe encoding the –58 to –13 region of the cPLA₂ promoter. Purified GST was used as a control. As shown in Figure 3(A), a single complex was detected with GST–LKLF (complex a); GST alone gave no higher migrating species (lane 4 versus lane 1). This band could be displaced by a 50-fold excess of unlabelled oligonucleotide (results not shown). EMSA analysis using a labelled oligonucleotide containing mutations at the –37/–30 position did not show a band with either GST or GST–LKLF (lanes 2 and 5). However, use of a labelled oligonucleotide containing mutations at –21/–18, which eliminates promoter activity, resulted in a band at the same position (lanes 3 and 6). To confirm that the complex observed with GST–LKLF contained LKLF, extracts were preincubated with an antiserum against LKLF (a gift from Dr J. Lingrel). In the presence of the antiserum, the GST–LKLF–DNA complex was partially shifted to a higher mobility complex (Figure 3B); this shift was not observed

in the presence of normal rabbit serum. These results indicate that LKLF binds to this region of the promoter, and can specifically associate with the –37/–30 region.

siRNA studies

Since overexpression of transcription factors can lead to artifactual results, we employed an siRNA strategy. Custom plasmids encoding for siRNA constructs specific for LKLF were obtained from GenScript Corporation. These encode hairpin structures that are processed to produce 22 bp siRNA oligonucleotides. RL-65 cells were transfected with plasmids encoding siRNA against LKLF, a scrambled siRNA, or empty vector (pU6) as a control. Stable clones were selected by growth in media containing G418. Individual clones were screened for expression of LKLF by immunoblotting with a specific LKLF antibody (Santa Cruz Biotechnology). In two independent clones (siRNA-LKLF-1 and -LKLF-2), LKLF expression as assessed by immunoblotting with a specific antibody was undetectable (Figure 4A, upper panel, lanes 4 and 5) compared with cells transfected with either empty vector (lanes 1 and 2) or scrambled siRNA (lane 3). Concomitantly, basal expression of cPLA₂ in these clones was markedly decreased (Figure 4A, lower panel). These effects on cPLA₂ expression were confirmed by direct measurement of enzyme activity using radiolabelled substrate. Activity using [¹⁴C]arachidonoyl phosphatidylcholine showed a 50% decrease in cPLA₂ activity (Figure 4B), consistent with the change in enzyme expression. To verify the role of LKLF in H-Ras-mediated induction of cPLA₂ promoter activity, cells stably expressing siRNA against LKLF or control cells stably transfected with empty vector were transiently co-transfected with the minimal region of the cPLA₂ promoter along with an expression construct for H-Ras. In both clones, where siRNA had decreased expression of LKLF, H-Ras induction of cPLA₂ promoter activity was inhibited by approx. 50% (Figure 4C). Finally, we examined the effect of blunting LKLF expression in NSCLC. Transient expression of siRNA against LKLF had no significant effect on steady-state cPLA₂

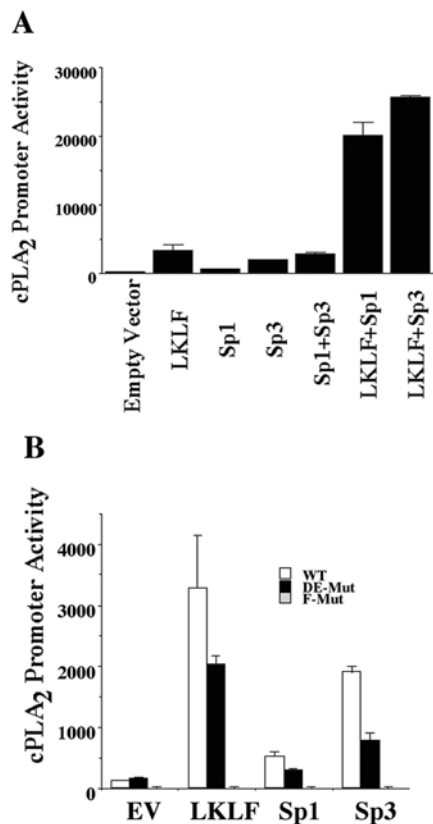


Figure 2 Effect of Krüppel family members on cPLA₂ promoter activity in *Drosophila* SL-2 cells

(A) *Drosophila* SL-2 cells were transfected with expression plasmids in the pACT2 vector encoding LKLF, Sp1, Sp3 or combinations of these factors, along with the cPLA₂ promoter construct. Cells were harvested after 72 h, and promoter activity normalized to protein was determined. (B) SL-2 cells were transfected with empty vector or with the expression plasmids encoding LKLF, Sp1 or Sp3, along with either the wild-type cPLA₂ promoter construct (WT), construct encoding mutations at -37/-30 (DE mut), or mutations at -21/-8 (F mut). Promoter activity normalized to protein was determined as in (A).

promoter activity. Similarly, an siRNA against Sp1, which decreases expression of this factor (results not shown) did not inhibit cPLA₂ promoter activity either. However, when both siRNA constructs were transiently expressed together, promoter activity was significantly decreased (Figure 4D).

In vitro phosphorylation of LKLF

Our previous studies have demonstrated that induction of cPLA₂ promoter activity by H-Ras is mediated by activation of the ERK and JNK MAP kinase pathways [21]. While the targets of these kinases, which are critical for transcriptional control of cPLA₂, have not been identified, one hypothesis is that these kinases directly phosphorylate transcription factors leading to their activation. We therefore examined whether LKLF is a substrate for ERKs. ERKs were activated by stimulating RL-65 cells for 5 min with EGF, and measuring kinase activity in immunocomplexes after immunoprecipitation with anti-ERK antibodies as described previously in [33]. Activation by EGF was confirmed using myelin basic protein as substrate (Figure 5, lanes 3 and 4). Incubation of immunocomplexes with GST-LKLF in the kinase reaction resulted in ³²P incorporation into a band of approx. 65 kDa, which co-migrated with GST-LKLF (Figure 5, lanes 5-8). Incorporation with EGF-stimulated ERKs was 3-4-fold more than control ERK

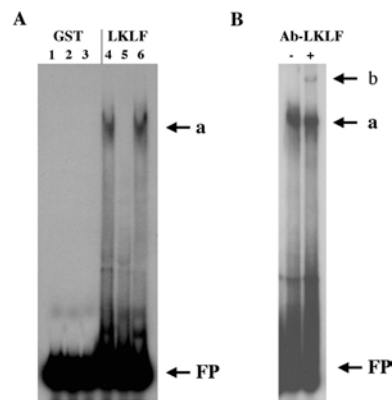


Figure 3 EMSA analysis of LKLF binding to the cPLA₂ promoter

(A) GST-LKLF was purified using glutathione-agarose; control containing GST alone was purified in parallel. Purified protein (~1 μg) was incubated with double-stranded ³²P-labelled probe corresponding to the -58 to +12 region of the cPLA₂ promoter and analysed on non-denaturing gels, followed by autoradiography. Lanes 1-3, GST proteins; lanes 4-6, GST-LKLF. Lanes 1 and 4 were incubated with wild-type probe; lanes 2 and 5 were incubated with a probe containing the Asp/Glu mutation (-37/-30) and lanes 3 and 6 were incubated with probe containing the Phe mutation (-21/-18). A single band was observed (a). (B) GST-LKLF was incubated with wild-type double-stranded labelled probe in the absence (-) or presence (+) of 1 μg of anti-LKLF-antibody. Presence of the antibody caused a partial shift of complex a to a slower migrating complex b.

immunoprecipitates. No incorporation of ³²P was detected using GST as a substrate. These results indicate that activated ERKs can phosphorylate LKLF *in vitro*, and suggest that LKLF may be a target of the ERK pathway mediating induction of cPLA₂.

DISCUSSION

Increased expression of cPLA₂ is observed in a variety of cancers and is critical for increased prostaglandin production associated with tumours. Inhibition of prostaglandin production by non-steroidal anti-inflammatory drugs is growth-inhibitory in lung cancer cells and in animal models of lung tumorigenesis, suggesting that induction of cPLA₂ is critical for tumorigenesis. Consistent with this finding, lung tumorigenesis is inhibited in mice that are deficient in cPLA₂ [36]. We have previously shown that expression of oncogenic Ras is both necessary and sufficient for cPLA₂ induction [12,21], and that this is mediated through transcriptional activation of the cPLA₂ promoter. The cPLA₂ promoter is a TATA-less promoter containing a GC-rich region in the proximal region that is highly conserved between humans, rat and mouse. We have previously reported that Sp1 can bind to this region and acts as a transcriptional activator [22]. However, the regulation of cPLA₂ can probably be combinatorial, involving multiple transcription factors that bind to this region.

LKLF or KLF-2 is a member of the Krüppel family of transcription factors, and was so-named originally because of its high expression in the lung, but also is expressed in the spleen, vasculature, heart, skeletal muscles, kidney, testis, uterus and other lymphoid organs [34,37]. Deletion of LKLF is embryo-lethal, but by using chimaeric mice, it was shown that LKLF is important for lung development [38]. It is also important in development of blood vessels [39] and the immune system [37]. In the present study, we have identified LKLF as an important regulator of cPLA₂ expression in lung epithelial cells and in NSCLC cell lines. While the one-hybrid screen used to identify LKLF can in principle isolate both repressors and activators of gene expression, our results support a role for LKLF as an activator of cPLA₂

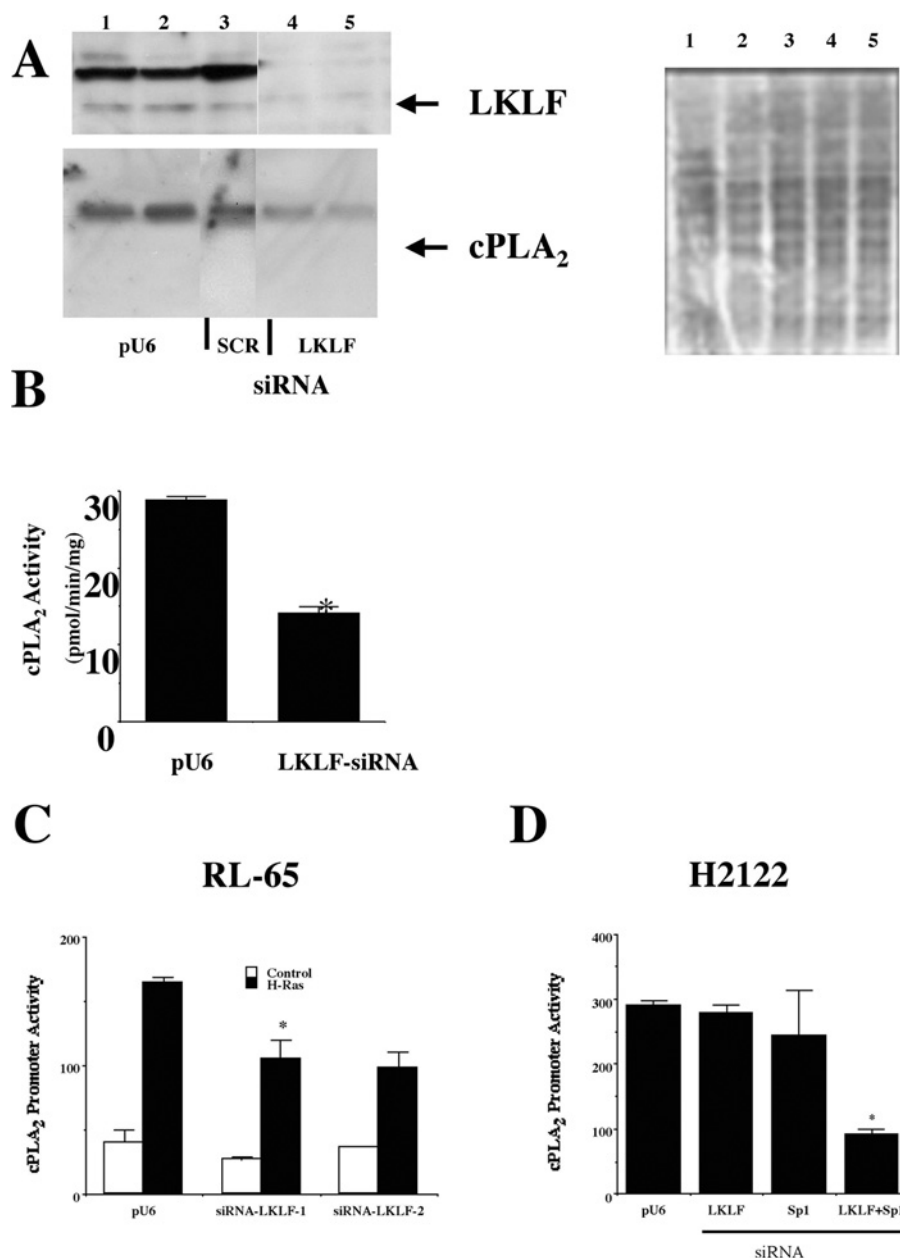


Figure 4 siRNA against LKLF inhibits cPLA₂ expression and promoter activity

(A) Individual clones of RL-65 cells, stably transfected with plasmid encoding an siRNA directed against LKLF (LKLF-1 and -2, lanes 4 and 5), with control empty vector (pU6, lanes 1 and 2), or with a scrambled LKLF siRNA (SCR, lane 3) were harvested and protein lysates prepared in RIPA buffer [50 mM Tris/HCl (pH 7.0), 150 mM NaCl, 1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 2 mM EDTA and 200 μ M Na₃VO₄]. Equal amount of lysate protein was immunoblotted for expression of LKLF (upper panel) or cPLA₂ (lower panel). Steady-state cPLA₂ expression was decreased in both clones expressing the LKLF siRNA compared with controls or cells transfected with scrambled siRNA. Loading control for the LKLF blot is shown on the right. (B) Extracts were prepared from pools of RL-65 cells, stably transfected with empty vector (pU6) or siRNA against LKLF after Dounce homogenization and ultracentrifugation as described previously in [16]. Enzyme activity was assayed using [¹⁴C]arachidonoyl-phosphatidylcholine in the presence of 5 mM Ca²⁺. (C) One of the control clones (pU6) and both clones expressing LKLF-siRNA (LKLF-1 and -2) were transiently transfected with the cPLA₂ promoter construct with or without an H-Ras expression plasmid. Promoter activity was determined 48 h after transfection. Both siRNA clones showed a decrease in H-Ras-induced promoter activity. (D) H2122 cells were transiently transfected with the cPLA₂ promoter construct along with the plasmids encoding siRNA for LKLF, Sp1 or both constructs together. Control cells were transfected with empty vector (pU6). Promoter activity normalized to β -galactosidase was measured after 48 h incubation. Both siRNA constructs significantly decreased steady-state promoter activity. * $P < 0.05$ versus pU6.

transcription and indicate that it plays an important role in the induction of this protein by gain-of-function Ras.

Overexpression of LKLF in normal lung epithelial cells was sufficient to increase cPLA₂ promoter activity, and expression of a dominant-negative form of the protein inhibited the induction of promoter activity by oncogenic Ras and decreased steady-state promoter activity in NSCLC cells which express gain-of-function

Ras. The relatively modest increase in promoter activity seen with overexpression in RL-65 cells can be attributed to the presence of multiple factors (e.g. Sp1), which can compete for binding and regulation of the promoter. We therefore used SL-2 cells that are devoid of Sp1 family members to assess the relative ability of different factors to regulate the cPLA₂ promoter. In these cells, LKLF was a potent stimulator of cPLA₂ promoter activity,

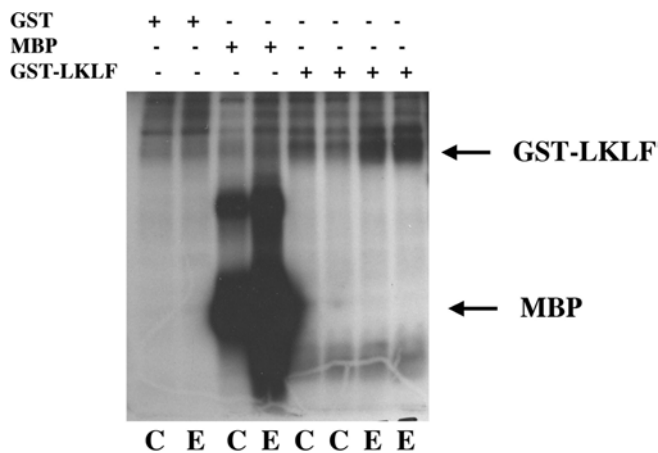


Figure 5 Phosphorylation of GST-LKLF by activated ERK

RL-65 cells were stimulated for 5 min with EGF (E) or vehicle (C), and lysates were prepared in MAP kinase lysis buffer. ERKs were immunoprecipitated with anti-ERK-1 and anti-ERK-2, and Protein A-Sepharose. After washing, the immunoprecipitates were resuspended and kinase activity using Mg[γ -³²P]ATP was determined using either recombinant GST, myelin basic protein or GST-LKLF (duplicate samples, as indicated). Samples were analysed by PAGE, followed by autoradiography.

increasing activity as least as well as either Sp1 or Sp3. Interestingly, expression of LKLF synergized with either Sp1 or Sp3 expression, while these two factors appeared additive. This suggests that, while Sp1 and Sp3 may act through a common element, LKLF may act through a different site. By EMSA analysis, it appears that LKLF binds to the -37/-30 region of the cPLA₂ promoter. We have previously shown that this region also binds Sp1 [22], further indicating that these factors may compete for binding to this region. Interestingly, mutations at the -21/-18 region of the promoter completely abolished both basal and H-Ras-induced promoter activity, but did not affect binding of LKLF. Based on analogies with other promoters, we would propose that this region represents the binding site for the transcription machinery, and specific transcription factors binding to upstream regions co-operate to initiate transcription.

It is therefore difficult to ascertain which members of the Krüppel family actually regulate cPLA₂ expression, since over-expressing one of these factors may increase promoter activity in an artificial manner. Results using siRNA (Figure 4) strongly support an important role for LKLF in this regulation. Expression of the siRNA to LKLF selectively blocked the expression of LKLF, without affecting Sp1 or Sp3 expression (results not shown). In this setting, steady-state cPLA₂ protein expression and activity are blunted, and induction of promoter activity by H-Ras is inhibited in normal lung epithelial cells. Studies in NSCLC, where expression of cPLA₂ is constitutively driven, appears to require co-operation between both LKLF and Sp1. Decreasing expression of either factor by siRNA did not significantly alter cPLA₂ promoter activity, but inhibiting expression of both factors severely decreased the expression. It is probable that the regulation of cPLA₂ expression in NSCLC is multifactorial, involving the co-operation of Ras effector pathways with other oncogenic pathways. Our earlier studies have implicated a role for the JNK and ERK pathways in mediating cPLA₂ induction. While the targets of these kinase pathways are yet to be identified, direct phosphorylation of transcription factors with concomitant activation is a possible model. Our results indicate that LKLF is a substrate for ERK. To definitely establish the role of LKLF phosphorylation in regulating cPLA₂ expression, mapping of the phosphorylation sites on the protein *in vivo*, followed by site-directed mutagenesis

of these phosphorylation sites to determine their role in Ras-mediated induction of the cPLA₂ promoter are required.

Increased cPLA₂ expression is critical for transformed growth of NSCLC. Exposure of these cells to a specific inhibitor of the enzyme, or blocking downstream production of prostaglandins with cyclo-oxygenase inhibitors, inhibits anchorage-independent growth of these cells [12]. However, these agents act on additional pathways. Decreasing expression of specific transcription factors such as LKLF may provide a novel therapeutic target for inhibition of tumorigenesis.

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