LKB1/STK11 Suppresses Cyclooxygenase-2 Induction and Cellular Invasion through PEA3 in Lung Cancer

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Abstract
We showed that the PEA3 transcriptional factor interacted with LKB1, a serine/threonine kinase, which is somatically mutated in lung cancer. This interaction occurred through the ETS domain of PEA3 and the kinase domain of LKB1. Mutation of LKB1 in lung cancer cells stabilized PEA3. Reintroduction of wild-type (WT) LKB1 into cells induced down-regulation of PEA3 and subsequently resulted in reduced cyclooxygenase-2 RNA and protein expression, whereas germ-line and somatic LKB1 mutants were defective in this activity. LKB1 phosphorylated PEA3 and promoted its degradation through a proteasome-mediated mechanism. Cells expressing mutant LKB1 possessed greater invasive potential compared with cells expressing WT LKB1. Increased invasion of cells with mutant LKB1 was partly due to PEA3 expression, as RNA interference inhibition of PEA3 resulted in decreased cyclooxygenase-2 RNA expression. These results suggest that PEA3 stabilization due to LKB1 inactivation could lead to epithelial/mesenchymal transition and greater lung cancer invasion potential. (Cancer Res 2006; 66(16): 7870-9)

Introduction
Germ-line mutations in the serine-threonine kinase LKB1/STK11 result in Peutz-Jeghers syndrome (PJS; refs. 1, 2). PJS is an autosomal inherited disorder characterized by intestinal hamartoma, oral mucocutaneous hyperpigmentation, and increased risk for gastrointestinal and extraintestinal malignancies (3–5). Mutations of LKB1 result in complete loss of LKB1 protein expression (2). LKB1/STK11 inactivation in primary lung adenocarcinomas and in lung cancer cell lines is a common event (6). Therefore, the formation of hamartomas and tumor in PJS is mediated by inactivation of the remaining wild-type (WT) allele. LKB1 heterozygous mice were recently shown to develop hepatocellular carcinoma and intestinal polyps (7, 8). Molecular characterization of the polyps showed that cyclooxygenase-2 (COX-2) was up-regulated through activation of extracellular signal-regulated kinases 1 and 2 (9). DNA chip microarray analysis of the mouse polyps (LKB1+/− versus LKB1−/−) has shown that LKB1 can modulate factors linked to angiogenesis, extracellular matrix remodeling, cell adhesion, and inhibition of Ras-induced transformation (7).

To elucidate the unknown molecular mechanism of LKB1-mediated tumor suppression, we applied a yeast two-hybrid approach to identify LKB1-interacting proteins. Here, we showed that LKB1 binds, phosphorylates, and down-regulates PEA3-mediated induction of COX-2 RNA and protein. In addition, we showed that RNA interference (RNAi) knockdown of PEA3 transcription decreased the invasive potential of lung cancer cells, whereas forced expression of PEA3 induced epithelial/mesenchymal transition of lung epithelial cells.

Materials and Methods

Plasmids. The cDNA for hemagglutinin (HA)-tagged WT LKB1 was a gift from Dr. M. Sanchez-Cespedes (Spanish National Cancer Center, Madrid, Spain). To generate HA-LKB1 mutants (D194A, KHStop, and Δ175-176) with impaired kinase activity [kinase dead (KD)], we used the QuikChange Mutagenesis kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). pcDNA3.1 FLAG-tagged PEA3 expression vector was a kind gift from Dr. J. Hassell (McMaster University, Hamilton, Ontario, Canada). The pGL3-COX-2 promoter-luciferase reporter plasmid was a gift from Dr. C.C. Harris (National Cancer Institute, Frederick, MD). The COX-2 promoter deletion constructs were amplified using PCR (list of primers is available on request) and then subcloned into the pGL3-basic vector using the MluI and XhoI restriction sites (Promega, Madison, WI).

Yeast two-hybrid screen and mapping. To screen for the LKB1-interacting protein candidates, we used the LexA-based system (10). The full-length LKB1 cDNA was introduced into the LexA expression vector (pEG202) using the EcoRI and NotI restriction sites in frame with and downstream to LexA-binding domain. Because of strong self-activation of the LexA-LKB1 bait, we deleted the COOH terminus of LKB1 (residues 301-433). This deletion eliminated self-activation activity, and we therefore used this COOH-terminal truncated LKB1 bait (residues 1-300) to screen a human prostate cancer two-hybrid cDNA library based on pG4-5 yeast expression vector (Origen Technologies, Rockville, MD). For activation domain plasmids harboring various deletions of PEA3, we used pG4-5. The full-length PEA3 cDNA was subcloned into the EcoRI and XhoI restriction sites of pG4-5, and serial internal deletions were made using the QuikChange Mutagenesis kit according to the manufacturer's protocol. The yeast two-hybrid screens were done essentially as described elsewhere (10).

Cell cultures, transfections, and reporter assays. Human embryonic kidney cell line HEK293, lung cancer cell lines A427, A549, H1395, H1299, and H2095, and normal human bronchiolar epithelial (NHBE) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and grown in the recommended medium under standard conditions.

The expression cassettes for LKB1 and PEA3 were transiently transfected into cells using FuGENE 6 reagent (Roche Molecular Biochemicals, Inc., Indianapolis, IN). DNA chip microarray analysis of the mouse polyps (LKB1+/− versus LKB1−/−) has shown that LKB1 can modulate factors linked to angiogenesis, extracellular matrix remodeling, cell adhesion, and inhibition of Ras-induced transformation (7).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Western blot analysis and immunoprecipitation. For protein extraction, 5 × 10^5 cells per well were plated into six-well plates and transiently transfected with 0.5 μg of pcDNA3.1 FLAG-PEA3 expression plasmid and 1.5 μg of pcDNA3.1-HA-LKB1 expression plasmid. Eighteen hours after transfection, cells were incubated with or without proteasome inhibitor MG-132 (40 μmol/L) for 6 hours before protein extraction. Protein extraction and Western blot analysis were done as described previously (13). Primary antibodies against PEA3 (1:500), LKB1 (1:500), and HA (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA), FLAG (1:2,0000) and β-actin (1:2,000), both from Sigma (St. Louis, MO), and COX-2 (1:500; Cayman Chemical, Ann Arbor, MI) were used for protein detection and immunoprecipitation. For immunoprecipitation, HEK293 cells transfected with the indicated expression cassettes were lysed in buffer [50 mmol/L Tris (pH 7.5), 300 mmol/L NaCl, 5 μmol/L aprotinin, pepstatin, 1% NP-40, 1 mmol/L EDTA, 0.25% deoxycholate]. Total cell lysates (500 μL) were incubated with 5 μg of primary antibodies against LKB1 or PEA3 for 16 hours at 4°C followed by subsequent incubation with anti-HA antibody conjugated to agarose beads (Roche) for 2 hours at 4°C, and the beads were then washed with lysis buffer and protein complexes were resolved by a 4% to 20% SDS-PAGE. Endogenous protein-protein interactions between PEA3 and LKB1 were examined in total cell lysate obtained from the breast cancer cell line Sk-Br3 (purchased from ATCC). For immunoprecipitation, lysates were incubated overnight with the indicated antibodies. Immune complexes were precipitated with protein A/G-Sepharose beads and washed with lysis buffer before being resolved on SDS-PAGE.

Immunofluorescence confocal imaging. For immunofluorescence microscopy, H1299 cells were grown on coverslips, fixed with 4% paraformaldehyde, and then incubated with anti-FLAG M2 monoclonal antibody (1:200) for 2 hours. Proteins were visualized by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse and anti-rabbit immunoglobulins (IgG; Jackson Immunoresearch, West Grove, PA). Finally, coverslips were incubated with Hoechst 33258 for 5 minutes and inspected with a laser scanning confocal microscope (Bio-Rad, Hercules, CA) at the Johns Hopkins University School of Medicine Confocal Imaging Core (Baltimore, MD).

Electrophoretic mobility shift assay. H1299 cells were resuspended in 10 mmol/L Tris-HCl (pH 7.5)/5 mmol/L MgCl2/0.05% (v/v) Triton X-100 and lysed with Dounce homogenizer. The homogenate was centrifuged at 3,000 × g for 15 minutes at 4°C. The nuclear pellet was resuspended in an equal volume of 10 mmol/L Tris-HCl (pH 7.4)/5 mmol/L MgCl2; followed by the addition of one nuclear pellet volume of 1 mol/L NaCl/0/10 mmol/L Tris-HCl (pH 7.4)/4 mmol/L MgCl2. The lysed nuclei were left on ice for 30 minutes and then spun down at 10,000 × g for 15 minutes at 4°C. The supernatant (nuclear extract) was kept, and protein concentration was measured with bicinchoninic acid protein assay (Pierce Biochemicals, Rockford, IL). Nuclear proteins (10 μg) were subjected to electrophoretic mobility shift assay (EMSA). An oligomer representing −140 to −52 of the COX-2 promoter (available on request) was radioactively labeled and used in EMSA analysis.

Northern blot analysis. Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA (10 μg) from cells was denatured and loaded on a 1% formaldehyde-agarose gel. The RNA was transferred onto a Nytran membrane (Schleicher & Schuell, Keene, NH) using the TurboBlotter (Schleicher & Schuell) in 20× SSC buffer and subsequently UV cross-linked in a Stratalinker (Stratagene). The membran was prehybridized and hybridized in 7 mL of hybridization buffer (0.25 mol/L NaHPO4 plus 7% SDS) with 32P-labeled full-length cDNA probes for either PEA3 or COX-2 overnight at 65°C in hybridization buffer at constant rotation. After hybridization, the membrane was washed twice (30 minutes each) in 20 mmol/L NaHPO4 and 5% SDS at 65°C and then washed twice in 20 mmol/L NaHPO4 and 1% SDS (30 minutes each) at 65°C. Finally, the membrane wrapped in plastic paper was exposed to X-ray film (Kodak, Rochester, NY).

Small interfering RNA design and manipulation. Small interfering RNA (siRNA) oligonucleotides for LKB1 and scrambled siRNA oligonucleotides were purchased from Dharmacon (Lafayette, CO) and used according to the manufacturer's recommendation. Briefly, control and LKB1 siRNA oligonucleotides (200 pmol/well/plate) were transiently transfected into H1299 lung cancer cells using FuGENE 6 (4 μL) and 24 hours later, total cell lysates were used for Western blot analysis as described above. For LKB1-RNAi plasmid, the oligonucleotide 5'-TCAAGATGCTTACGCTTGTTGGAAGATGTCTGACAGACATTTTTT-3' and its reverse complement with overhanging XbaI site were cloned into the SalI and XbaI site of the pSuppressor/Neo vector (Imagenex, San Diego, CA). The cloned oligonucleotide was sequenced to confirm the absence of mutations occurring during the cloning process.

Cell invasion/Matrigel assay. Cells (1 × 10^5) in 0.5 mL of serum-free MEM were added to each well of 24-well/8-μm pore invasion membrane chambers coated with Matrigel (BD Discovery Labware, Bedford, MA). The lower chambers contained 10% fetal bovine serum (FBS) in MEM to serve as a chemoattractant. Cells were allowed to migrate or invade over the course of 48 hours. Cells that failed to penetrate the filters were removed by scrubbing with cotton swabs. Chambers were fixed with 100% methanol for 2 minutes, stained with 0.5% crystal violet for 2 minutes, rinsed in water, and examined under a bright-field microscope. Values for invasion and migration were obtained by counting five fields per membrane (20× objective) and represented the average of three independent experiments done over multiple days.

In vitro kinase assay. Active His-tagged LKB1 protein (purified from recombinant baculovirus-infected SF21 cells) was purchased from Upstate Cell Signaling Solutions (Waltham, MA). This enzyme (His-tagged LKB1, 51 kDa) is provided as a complex with glutathione S-transferase (GST)-MO25a protein (66 kDa) serving as a control substrate for LKB1. Both proteins were phosphorylated by incubation with a kinase mixture. For bacterial expression, the full-length PE3 cDNA was subcloned into the BamHI and XhoI sites of the pGEX-5X-2 bacterial expression vector (Amersham/Pharmacia, Piscataway, NJ). The resulting expression cassette, pGEX-5X-2-PEA3, was used to generate PE3 point mutants (T335A, T336A, S393A, and S395A) using the QuikChange Mutagenesis kit according to the manufacturer's protocol. WT and point mutants of GST-PEA3 were purified from Escherichia coli using a GST MicroSpin Purification Module (Amersham/Pharmacia). Purified GST-PEA3 (0.1 μg) and LKB1 (0.1 μg) were mixed and incubated for 30 minutes at 30°C in kinase buffer (50 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.1% 2-mercaptoethanol, 5 mmol/L MgCl2, 0.4 mmol/L MnCl2, 75 μg/mL bovine serum albumin, 20 μL/mL ATP, 10 μL of [γ-32P]ATP). Samples were separated by SDS-PAGE and visualized by autoradiography.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) was done using a ChIP assay kit (Upstate Cell Signaling Solutions). H1299 cells (2 × 10^6) were transfected with mock (pcDNA3.1), pcDNA3.1 FLAG-PEA3, or pcDNA3.1 FLAG-PEA3 plus pcDNA3.1-LKB1 and cross-linked with 1% formaldehyde, and ChIP was done according to the manufacturer's protocol. After reversing the cross-links, chromatin was purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) and samples were eluted with 30 μL of elution buffer. Chromatin was immunoprecipitated with 1 μg of anti-FLAG antibody M2 (Sigma). Immunoprecipitates were used as templates for PCR amplification of the COX-2 promoter with the following primers: COX-2, 5'-TGTTTCCGATTTTCT-3' and its reverse and samples were eluted with 30 μL of elution buffer. Chromatin was immunoprecipitated with 1 μg of anti-FLAG antibody M2 (Sigma). Immunoprecipitates were used as templates for PCR amplification of the COX-2 promoter with the following primers: COX-2, 5'-GCTTCCTG-GGTTCAGATTCTTCT-3' (sense) and 5'-GGTTAGCTTGGCCTGTCAG-3' (antisense). PCR was done as follows: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes using Platinum Taq I DNA polymerase (Invitrogen) for 37 cycles.
**Tissue specimens.** Human tissue samples were obtained from paraffin-embbeded blocks from the tissue bank of the Department of Pathology at the Johns Hopkins School of Medicine (approved by the Joint Committee on Clinical Investigation). Lung frozen tissues were obtained from Dr. William Westra (Johns Hopkins University). Lung samples were homogenized in ice-cold nuclear buffer [150 mmol/L NaCl, 1 mmol/L KH2PO4 (pH 6.4), 1 mmol/L EGTA, 5 mmol/L MgCl2, complete mini protease cocktail ([Roche]) at 4°C. Cels were then exposed to 0.3% Triton X-100, and nuclear pellets were spun down at 3,500 rpm for 20 minutes at 4°C. The nuclear pellets were mixed gently with an equal volume of nuclear buffer containing 0.4 mol/L NaCl at 4°C and spun down at 10,000 rpm for 20 minutes at 4°C. Protein concentration in supernatants was determined before Western blot analysis.

**Results**

**LKB1 associates with PEA3 in yeast.** To characterize the molecular mechanism underlying the role for LKB1 in tumor suppression, we searched for LKB1-interacting proteins using the LKB1 kinase domain (residues 1-306) as bait. Because the LexA plasmid harboring the full-length LKB1 displayed strong self-activation activity, we deleted the COOH-terminal regulatory domain of LKB1 (residues 301-433). The resulting LKB1 COOH-terminal truncated bait was used to screen the two-hybrid cDNA library from human prostate cancer cells. From this screen, we identified human prostate-derived ETS factor (PDEF) as a highly probable LKB1-interacting partner. A homology search showed that PDEF has similarities to PEA3 belonging to the ETS family of transcriptional factors (14–16) and a known regulator of COX-2. To determine whether PEA3 interacts with LKB1, we cloned PEA3 into the prey plasmid pG4-5 (activation domain) and did a yeast two-hybrid analysis using the LKB1 COOH-terminal truncation subcloned into the pEG202 plasmid (binding domain). As shown, the LKB1 COOH-terminal truncation (containing kinase domain) indeed physically associated with PEA3 (Supplementary Fig. S1).

We further mapped the interacting domains of both LKB1 and PEA3 using serial deletion of both proteins and yeast two-hybrid analysis (Fig. 1). We found that the ETS domain of PEA3 is absolutely necessary for the interaction with LKB1 (Fig. 1B). Deletion of the activation and COOH-terminal domains of PEA3 significantly reduced its interaction with LKB1, suggesting that these domains may cooperate with the ETS domain in transactivation. We also observed that mutations in the activation domain or the COOH-terminal domain of PEA3 dramatically reduced the interaction between PEA3 and LKB1 possibly due to significant changes in the tertiary structure of these PEA3 mutants (Fig. 1B).

To further examine the association of LKB1 and PEA3, we did coimmunoprecipitation of ectopically expressed proteins, HEK293 cells were transiently transfected with the HA-tagged LKB1 and FLAG-tagged PEA3 expression cassettes. Protein complexes were precipitated with the indicated antibodies and resolved electrophoretically followed by immunoblotting with antibodies to LKB1 or PEA3 (Fig. 1A). We found that LKB1 binds PEA3 through
residues 150 to 200 that include the kinase domain (Fig. 1A). To explore the endogenous protein complexes between LKB1 and PEA3, we used Sk-Br3 breast cancer cells that express both LKB1 and PEA3. We observed that LKB1 was specifically precipitated with anti-PEA3 antibody but was not precipitated with irrelevant IgG (Fig. 1C). Reciprocally, anti-LKB1 antibody was able to immunoprecipitate PEA3 (Fig. 1C). To further analyze the PEA3/LKB1 association, we examined colocalization of these two proteins using confocal fluorescence imaging. H1299 lung cancer cells were transiently transfected with the green fluorescent protein (GFP)-LKB1 and FLAG-PEA3 fusion expression cassettes. Cells were stained for both GFP and FLAG markers with the indicated antibodies (Fig. 1D). Localization of LKB1 seems to be mostly nuclear, whereas PEA3 is localized in both nuclei and cytoplasm. Merging of these images clearly indicated that LKB1 and PEA3 colocalized (Fig. 1D, merge). These results suggest that LKB1 interacts with PEA3 in vitro and in vivo.

Functional relevance of the LKB1 and PEA3 interaction. To explore the functional relevance of the LKB1 and PEA3 interaction, we studied expression of PEA3 and COX-2 using Northern blot analysis with RNA from several cells expressing WT and mutant LKB1 with COX-2 (A) and PEA3 (B) cDNA probes. C, total cell lysates were analyzed by Western blot analysis for LKB1 protein expression, which shows decreased expression in LKB1-mutant cells. Increased COX-2 and PEA3 RNA expression is seen in all the LKB1-mutant lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. D, increased expression of PEA3 and COX-2 in lung tumor tissues expressing mutant LKB1. Total tumor lysates from eight specimens were prepared as described in Materials and Methods. Levels of PEA3 and COX-2 were measured by Western blot as described previously. Left 4 and right 4 lanes, lysates from tissues expressing WT and mutant LKB1, respectively.

Figure 2. Differential expression of PEA3 and COX-2. Northern blot analysis with RNA from several cells expressing WT and mutant LKB1 with COX-2 (A) and PEA3 (B) cDNA probes. C, total cell lysates were analyzed by Western blot analysis for LKB1 protein expression, which shows decreased expression in LKB1-mutant cells. Increased COX-2 and PEA3 RNA expression is seen in all the LKB1-mutant lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. D, increased expression of PEA3 and COX-2 in lung tumor tissues expressing mutant LKB1. Total tumor lysates from eight specimens were prepared as described in Materials and Methods. Levels of PEA3 and COX-2 were measured by Western blot as described previously. Left 4 and right 4 lanes, lysates from tissues expressing WT and mutant LKB1, respectively.

expression of LKB1. Therefore, we examined expression of LKB1 in WT and mutant cells that have been studied previously (6, 17) and found that indeed LKB1 protein expression was reduced in mutant cells compared with LKB1 WT cells (Fig. 2C). Finally, we examined the expression of PEA3 and COX-2 in lung cancer tissues expressing WT and mutant LKB1. These tissues have been characterized for LKB1 status previously (6). Protein was extracted from these tissues and Western blotted with PEA3 and COX-2-specific antibodies. Results showed that, overall, tissues with LKB1 mutation showed greater expression of PEA3 and COX-2 (Fig. 2D). These results further support our hypothesis that mutation in LKB1 leads to increased expression of PEA3 and COX-2 in vivo.

Therefore, we examined the effect of LKB1 on COX-2 promoter activity. Lung cancer A427 cells were transiently transfected with the pGL3-COX-2 luciferase reporter plasmid and Renilla luciferase plasmid along with LKB1 and PEA3 expression cassettes. We found that WT LKB1 significantly reduced COX-2 reporter activity (Fig. 3A). However, LKB1 mutations found in lung adenocarcinoma (K44Ter; ref. 6) and PJS polyps (Δ175-176; ref. 18) were much less effective in down-regulating the COX-2 reporter activity (Fig. 3A). Similar results were obtained in H1299 lung cancer cells expressing WT LKB1 (Supplementary Fig. S2). Moreover, we found that WT LKB1 was able to down-regulate COX-2 reporter activity induced by exogenous PEA3 (Supplementary Fig. S2). The KD LKB1 mutant was completely ineffective in down-regulating the COX-2 reporter activity in both A427 and H1299 cells (Fig. 3A; Supplementary Fig. S2).
To determine the regulatory elements in the COX-2 promoter that were responsible for PEA3-mediated induction, a series of the COX-2 promoter deletion constructs were introduced into A427 cells and luciferase reporter activity was measured (Fig. 3B). We observed that PEA3-induced COX-2 promoter activity was completely abolished with the \( /C0\)\(52/+59\) construct, suggesting that the sequence(s) critical for the promoter induction probably lies between \(/C0\)\(140\) and \(/C0\)\(52\). Furthermore, PEA3 was able to induce luciferase activity from a plasmid containing only the sequence \(/C0\)\(140\) to \(/C0\)\(52\) of the COX-2 promoter (Fig. 3C). Moreover, cotransfection of the WT LKB1 expression cassette into cells significantly decreased the COX-2 promoter activity, suggesting that down-regulation of COX-2 promoter by LKB1 is probably through PEA3 (Fig. 3C).

To further explore the effect of LKB1 on PEA3 activity, we did an EMSA using the sequence \(/C0\)\(140\) to \(/C0\)\(223\) of the COX-2 promoter. We found that LKB1 was able to reduce the DNA-binding activity of PEA3 under these experimental conditions (Fig. 3D). We also showed that the PJS-associated LKB1 mutant (SL8) with an intact kinase domain but impaired regulatory domain dramatically reduced the DNA-binding activity of PEA3, suggesting that the kinase domain of LKB1 is critical for inhibiting PEA3 activity (Fig. 3D).

To further understand the molecular interaction between LKB1 and PEA3 on the COX-2 promoter, we hypothesized that LKB1 might promote PEA3 degradation. We thus examined the effect of LKB1 on both exogenous and endogenous PEA3 protein levels. We found that introduction of WT LKB1 into A427 cells (expressing mutant LKB1) led to a marked decrease in the amount of PEA3 protein level (Fig. 3A).

**Figure 3.** Down-regulation of COX-2 transcription by LKB1. A, A427 cells (1 \(\times\) \(10^5\) per well, 24-well plate) were cotransfected with 0.1 \(\mu\)g of the pGL3-COX-2 promoter-luciferase plasmid, 0.25 \(\mu\)g of the pCDNA3.1 FLAG-PEA3 expression plasmid, and 0.75 \(\mu\)g of pcDNA3.1-HA-LKB1 (WT or mutant) expression plasmid into each well. To normalize data for transfection efficiency, cells were also transfected with 200 ng of the pRL-TK plasmid (Renilla luciferase). The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Twenty-four hours after transfection, luciferase activity was monitored using the Dual-Luciferase Assay kit in a Monolight TM-20 luminometer for 10 seconds. Three independent transfections were done, and calculated mean and SD were used for data presentation. WT LKB1 inhibited COX-2 promoter-driven luciferase reporter activity, whereas mutant LKB1 failed to do so. Statistically significant differences between data points were determined using a Student’s two-tailed \(t\) test. *, \(P_s < 0.05\). B, schematic representation of promoter deletions used to map the PEA3 regulatory element in the COX-2 promoter. C, A427 cells were cotransfected with equivalent amount of pCDNA3.1 FLAG-PEA3 or pCDNA3.1 FLAG-PEA3 + pcDNA3.1-HA-LKB1 (WT). All cells were also transfected with the pRL-TK plasmid and one of the pGL3-COX-2 promoter deletion constructs. All COX-2 deletion constructs were effectively inhibited by WT LKB1. Statistically significant differences between data points were determined using a Student’s two-tailed \(t\) test. *, \(P_s < 0.05\). D, down-regulation of PEA3 DNA-binding activity by LKB1. Transfection of H1299 cells with pCDNA3.1 FLAG-PEA3 induced a specific DNA-PEA3 complex formation, which is inhibited with WT but not an LKB1 KD mutant. Excess unlabeled oligomer (CC) was used as a competitor and effectively competed away the DNA-PEA3 complex. E, ChIP assay of PEA3 binding to the COX-2 promoter and its modulation by LKB1. PCR was done to amplify regions of the COX-2 promoter around the PEA3-binding sites.
at the Thr172 (19), confirming that Sk-Br3 cells indeed express WT downstream target for WT LKB1] led to phosphorylation of AMPK drug activating AMP-activated protein kinase (AMPK), the amino-4-imidazolecarboxamide riboside [AICAR; a cell-permeable identified that treatment of Sk-Br3 breast cancer cells with 5-of PEA3 and COX-2 in WT LKB1 genetic background. First, we endogenous LKB1, we screened for cells with high protein levels of PEAs and COX-2 in WT LKB1 genetic background. We found that treatment of Sk-Br3 breast cancer cells with 5-lysates were analyzed for COX-2, PEA3 and COX-2 expression (Fig. 4B, 2 and 3). We observed that a pharmacologic activation of LKB1 in Sk-Br3 cells resulted in abrogation of PEA3 and COX-2 expression (Fig. 4B, 2 and 3).

Next, we examined the role of the proteasome-dependent pathway in LKB1-mediated degradation of PEA3. We found that the 26S proteasome inhibitor MG-132 dramatically abrogated down-regulation of PEA3 and possibly ubiquitination of PEA3 in the presence of WT LKB1 (Fig. 4C and D). However, the pan-caspase inhibitor failed to inhibit LKB1-induced PEA3 degradation (Fig. 4C). To further examine ubiquitination of PEA3, we used H1299 cells transiently transfected with FLAG-tagged PEA3 alone or together with HA-tagged LKB1 (Fig. 4D). Twenty-four hours after transfection, PEA3 was immunoprecipitated with anti-FLAG antibody and resolved by SDS-PAGE. Western blot analysis with antibody against ubiquitin revealed a ladder of PEA3-specific high molecular weight bands, indicating ubiquitination of PEA3 (Fig. 4D).

To further examine the role of LKB1 in regulation of levels of PEA3 and COX-2, we used siRNA specific to LKB1 to selectively knock down its expression. We found that introduction of LKB1 siRNA into H1299 cells expressing WT LKB1 resulted in 80% reduction in the LKB1 protein level after 48 hours after transfection (Fig. 5). siRNA-mediated knockdown of LKB1 was accompanied by a dramatic increase in both PEA3 and COX-2 protein levels (Fig. 5), whereas scrambled siRNA oligonucleotides (control) had no effect (Fig. 5A). These results were confirmed using a vector-based LKB1 siRNA (LKB1-RNAi4; Fig. 5B), which showed a similar effect.

Because KD LKB1 was unable to down-regulate PEA3-induced COX-2, we explored the possibility that PEA3 protein degradation is associated with phosphorylation events induced by LKB1. To test this hypothesis, we did an in vitro kinase assay with GST-PEA3 as a substrate. AMPK, a well-known LKB1 substrate, was used as a positive control. We found that indeed LKB1 phosphorylated PEA3 and AMPK (Fig. 6A). The DNA-binding domain of PEA3 seemed necessary for its association with LKB1. Analysis of this domain in PEA3 revealed two threonine (Thr, T) and two serine (Ser, S) residues at amino acid 355, 363, 393, and 395, respectively.

Figure 4. LKB1 down-regulates endogenous COX-2 and PEA3 expression. A, A427 cells were transfected with the WT or mutant LKB1 expression cassettes (pcDNA3.1-HA-LKB1) for 24 hours. Total lysates were analyzed for COX-2, PEA3, and β-actin by Western blot analysis. Mutant LKB1 was less effective in decreasing PEA3 and COX-2 expression than WT LKB1. B, pharmacologic activation of LKB1 with a cell-permeable drug activating AMP-activated protein kinase (AMPK), the amino-4-imidazolecarboxamide riboside [AICAR; a cell-permeable identified that treatment of Sk-Br3 breast cancer cells with 5-

**Figure 4.** LKB1 down-regulates endogenous COX-2 and PEA3 expression.

A, A427 cells were transfected with the WT or mutant LKB1 expression cassettes (pcDNA3.1-HA-LKB1) for 24 hours. Total lysates were analyzed for COX-2, PEA3, and β-actin by Western blot analysis. Mutant LKB1 was less effective in decreasing PEA3 and COX-2 expression than WT LKB1. B, pharmacologic activation of LKB1 with 1 mmol/L AICAR for 12 hours, and total extracts were immunoblotted for phosphorylated AMPK (P-AMPK), PEA3, COX-2, and β-actin. Phosphorylated AMPK is a positive control. We found that indeed LKB1 phosphorylated PEA3 and AMPK (Fig. 6A). The DNA-binding domain of PEA3 seemed necessary for its association with LKB1. Analysis of this domain in PEA3 revealed two threonine (Thr, T) and two serine (Ser, S) residues at amino acid 355, 363, 393, and 395, respectively.
Therefore, we mutated each of these residues to alanine and did an
in vitro kinase assay with these PEA3 mutants. We found that LKB1
was able to phosphorylate PEA3 mutants (T355A, T363A, and S393A; Supplementary Fig. S4). However, LKB1 failed to
phosphorylate PEA3 mutant (S395A) (Fig. 6 A). To verify
that phosphorylation of Ser 395 of PEA3 is important for its
degradation by LKB1, we introduced expression cassettes for the
PEA3-mutant S395A and WT LKB1 into H1299 lung cancer cells.
Western blot analysis showed that the PEA3-mutant S395A could
not be degraded by LKB1. We also found that the PEA3 point
mutants (S393A and S395A; Fig. 6B) had greater protein stability
compared with WT PEA3.

PEA3 mediates cellular invasion and epithelial/mesenchy-
mal transition. COX-2 overexpression in lung cancer tissue has
been frequently associated with increased invasion (20). To further
assess the role of LKB1 in cellular invasion, we did the Matrigel
invasion assay using lung cancer cell lines A427 (expressing mutant
LKB1) and H1299 (expressing WT LKB1). We studied whether and
how these cells respond to chemoattractant (10% FBS) >48 hours.
We observed that A427 cells showed a higher degree of
invasiveness compared with H1299 cells (Fig. 7A). However, forced
expression of WT LKB1 in A427 cells led to ~8-fold reduction in
Matrigel invasion after 48 hours (Fig. 7A).

Next, we inhibited PEA3 transcription by stable siRNA
expression in A549 cells (expressing mutant LKB1 and high levels
of PEA3). We selected one stably transfected clone (clone 8)
showing a ~60% decrease in PEA3 protein level (Fig. 7A). Interestingly, this clone also showed ~50% decrease in COX-2
expression compared with control cells, further confirming a role
for PEA3 in COX-2 expression (Fig. 7A). We also showed that
clone 8 was ~40% less invasive in Matrigel assay than control
cells (Fig. 7A).

Several studies have shown a correlation between the epithelial/
mesenchymal transition and cancer invasion (21). It is well known
that the epithelial/mesenchymal transition is associated with
reduction in epithelial markers and increase in mesenchymal
markers (22). We examined whether expression of PEA3 was
able to promote an epithelial/mesenchymal transition in normal
mammalian cells. We thus expressed PEA3 in NHBE epithelial
cells. We found that forced expression of PEA3 resulted in
decrease in epithelial markers (e.g., E-cadherin, β-catenin, and
γ-catenin). In contrast, the expression of fibroblast markers

Figure 5. Two independent siRNA knockdown of LKB1 induces PEA3 expression. A, H1299 cells were transfected with the LKB1 siRNA oligo or control (scrambled) siRNA. B, similarly, LKB1-siRNA4 or empty vector (pSup) was transfected in H1299. Total lysates were analyzed for protein levels of LKB1, PEA3, COX-2, and β-actin. An increase in PEA3 and COX-2 expression is seen after LKB1 inhibition.

Figure 6. LKB1 phosphorylates PEA3 in vitro on Ser

A, purified active LKB1 protein (produced by recombinant
baculovirus-infected Sf21 cells) was tested for its ability to
phosphorylate the bacterially expressed and purified
His-tagged AMPK protein (Neumann et al., 2003) and
GST-MO-25 protein (Upstate Cell Signaling Solutions)
serving as positive controls. The in vitro kinase reaction
was done with the following incubation mixtures:
His-tagged LKB1 + GST-MO-25 (LKB1 + GST),
AMPK + GST, His-tagged LKB1 + AMPK, His-tagged
LKB1 + GST-PEA3 (with S395A mutation: LKB1 + S395A),
and His-tagged LKB1 + GST-PEA3. Right, phosphorylated
PEA3 band. LKB1 is unable to phosphorylate the PEA3
S395A mutant. B, H1299 cells were cotransfected with WT
or mutant FLAG-PEA3 and LKB1. Total cell extracts were
analyzed by Western blotting using antibodies against
FLAG and β-actin.
(e.g., fibronectin, vimentin, and smooth muscle actin), whose expression has been shown to correlate positively with the epithelial/mesenchymal transition (22), was strongly induced by PEA3 overexpression (Fig. 7B). These results suggest that overexpression of PEA3 can induce epithelial/mesenchymal transition in normal human epithelial cells and mediate increased cellular invasion of lung cancer cells.

**Discussion**

In this study, we provide evidence that PEA3, an ETS family transcription factor, physically and functionally interacts with the PJS tumor suppressor LKB1. First, our yeast two-hybrid assay indicated to us that PDEF bound LKB1 and would likely bind similar transcription factors. Moreover, COX-2 was implicated as an important target of LKB1 inactivation in human colon polyps. In an effort to tie in these two observations, we proceeded with a homology search to PDEF and discovered that PEA3 was a closely related and a relevant transcription factor for COX-2. This approach allowed us to link an important mediator of COX-2 with LKB1 inactivation. Second, we determined that binding to LKB1 is mediated by DNA-binding domain of PEA3, whereas interaction with PEA3 is mediated by the kinase domain of LKB1. Third, we observed that the physical association with LKB1 induced phosphorylation of PEA3 at S395 and targeted PEA3 into a ubiquitination and proteasome-dependent degradation pathway. Fourth, we found that LKB1-mediated degradation of PEA3 led to transcriptional down-regulation of COX-2 gene expression. Finally, we showed that LKB1-mediated degradation of PEA3 reduced invasiveness of lung cancer cells and forced expression of PEA3 induced an epithelial/mesenchymal transition of normal epithelial cells.

This work thus provides a mechanistic link between LKB1 tumor suppressor and PEA3 transcriptional activation of COX-2 gene expression. As a member of the ETS transcription factor family, PEA3 was shown to play a role in tumor progression. PEA3 was shown to be up-regulated in human breast cancer (14) and primary and metastatic lesions of mouse mammary carcinoma (23). Moreover, PEA3 protein expression predicts worse overall survival in breast cancer (24). Forced expression of PEA3 results in enhanced motility and invasion of non–small cell lung cancer cells (25). PEA3 factors are also shown to be highly expressed in tumors from Wnt1 transgenic mice, in which COX-2 is also up-regulated (26, 27).

Deregulation of COX-2 signaling is ubiquitous in human cancers (28, 29). COX-2, an inducible enzyme involved in prostaglandin biosynthesis, is overexpressed in several epithelial malignancies, including breast, prostate, lung, colorectal, esophageal, and ovarian human cancers (30–34). It was shown that COX-2 overexpression in human cancer cells enhances cell motility and invasiveness, thus suggesting a mechanism of COX-2-mediated metastasis. COX-2 is overexpressed in PJS hamartomas (35) and lung adenocarcinomas (36). The COX-2 overexpression was also associated with shortened survival in patients with resected early-stage adenocarcinoma of the lung (36). COX-2 inhibition decreases tumor cell proliferation in vivo and has been shown to enhance tumor radiosensitivity (37).

**Figure 7.** LKB1 and PEA3 regulate the invasion potential of lung cancer cells. A, equal numbers (10^5 cells) of H1299 cells (harboring WT LKB1) or A427 cells (harboring mutant LKB1) and A427 cells transfected with the WT LKB1 were seeded to the upper wells of Matrigel-coated chambers in serum-free medium. A549 cells harboring scramble (control) siRNA or PEA3 siRNA cells were added to the upper wells of Matrigel-coated chambers. For a chemoattractant, 10% FBS in MEM was placed in the bottom wells. After incubation for 48 hours, the remaining cells in the upper chamber were removed by scrubbing with cotton swabs and cells in the lower chambers were stained in 0.5% crystal violet. Photomicrographs were taken with a microscope using a 20× objective. 3 and 5, introduction of either the WT LKB1 or PEA3 RNAi dramatically suppressed invasion. Statistically significant differences between data points were determined using a Student’s two-tailed t test. *, P < 0.05. B, expression of epithelial proteins (e.g., E-cadherin, β-catenin, and γ-catenin) and mesenchymal proteins (e.g., fibronectin, vimentin, and smooth muscle actin [Sm-actin]) was examined by immunoblotting in NHBEC cells expressing either mock pcDNA3.1 or pcDNA3.1 FLAG-PEA3 cassettes. The pattern is entirely consistent with induction of an epithelial/mesenchymal transition. β-Actin was used as a loading control.

(e.g., fibronectin, vimentin, and smooth muscle actin), whose expression has been shown to correlate positively with the epithelial/mesenchymal transition (22), was strongly induced by PEA3 overexpression (Fig. 7B). These results suggest that overexpression of PEA3 can induce epithelial/mesenchymal transition in normal human epithelial cells and mediate increased cellular invasion of lung cancer cells.
Forced expression of COX-2 in cancer cells produces prostaglandins and proangiogenic factors [e.g., vascular endothelial growth factor (VEGF)], whereas aspirin and antibodies against VEGF abrogate angiogenesis (38). COX-2 overexpression has been shown in PJS (39) and in LKB1 heterozygous mice (9), suggesting a role for LKB1 in regulation of COX-2.

In our study, we showed that ectopic expression of LKB1 suppressed cellular invasive lung of cancer cells. In addition, cells expressing mutant LKB1 (A427T) showed greater invasive potential compared with cells expressing WT LKB1 (H1299), although no difference was found in the rate of cellular proliferation. Clearly, pathways that increase cellular invasion do not necessarily increase cellular proliferation, although interactions between these pathways cannot be excluded. LKB1 has been shown to regulate mammalian target of rapamycin, which plays a role in cancer development and progression. Therefore, it is expected that mutations in LKB1 lead to activation of multiple pathways that play a role in cellular invasion. Our proposed mechanism detailing the role of LKB1 in regulating PEA3-mediated invasion is one component of this machinery as pointed out by the author. Further validation of these results will require studies in more cell lines, in vivo studies, and additional observations on LKB1 downstream effects, which are outside the scope of this present study.

Invasive potential of LKB1-mutant cells is, at least in part, mediated by PEAM because most cells with down-regulation of PEAM levels showed decreased cellular invasion and activation of epithelial/mesenchymal transition. PEAM expression in human cancers (e.g., breast tumors) was found to correlate with down-regulation of E-cadherin and increased expression of matrix metalloproteinases (MMP-2 and MMP-7) and increased invasiveness of cancer cells (16). E-cadherin is critical for maintenance of cell polarity and differentiation (40). However, a decrease in E-cadherin expression leads to defects in cell-cell adhesion and, therefore, to tumor cell dissemination (41).

There is also evidence that K-Ras may regulate COX-2. Mutations in K-Ras are found in ~30% of lung adenocarcinomas and associated with poor survival (42). Human non–small cell lung cancers with mutations in K-Ras have high expression of COX-2 (43). Expression of mutant K-Ras activates the Raf/mitogen-activated protein kinase pathway and results in increased transcription of COX-2 (44). Oncogenic K-Ras also activates the phosphatidylinositol 3-kinase pathway and results in post-transcriptional stabilization of COX-2 mRNA (45). LKB1 and K-Ras pathways are most likely additive because several lung cancer cell lines harbor mutations in both of these genes (17). Taken together, LKB1 or K-Ras mutation in lung cancer can lead to PEAM and COX-2 up-regulation, resulting in resistance to apoptosis, increased angiogenesis, and increased invasion and metastasis. Because PEAM is a major transcriptional regulator of COX-2 expression and probably other oncogenic targets in lung adenocarcinomas, PEAM itself in addition to COX-2 may be a viable therapeutic target for human cancers with LKB1 mutations. In addition to in vitro and clinical studies of COX-2 inhibitors in colon polyps, nimesulide (a COX-2 inhibitor) can inhibit proliferation of non–small cell lung cancer cell lines (38). Our data suggest that similar approaches deserve further attention, particularly in lung cancers with LKB1 mutations.

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15. Bieche I, Tozlu S, Girault I, et al. Expression of mutant K-Ras activates the Raf/mitogen-activated protein kinase pathway and results in increased angiogenesis, and increased invasion and metastasis. Because PEAM is a major transcriptional regulator of COX-2 expression and probably other oncogenic targets in lung adenocarcinomas, PEAM itself in addition to COX-2 may be a viable therapeutic target for human cancers with LKB1 mutations. In addition to in vitro and clinical studies of COX-2 inhibitors in colon polyps, nimesulide (a COX-2 inhibitor) can inhibit proliferation of non–small cell lung cancer cell lines (38). Our data suggest that similar approaches deserve further attention, particularly in lung cancers with LKB1 mutations.
Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to \( \text{O}_2 \) consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 (+0.27) + 0.35 (-0.16) = +0.12 ,
\]

a figure identical to the observed +0.12 for normal leukocytes.
LKB1/STK11 Suppresses Cyclooxygenase-2 Induction and Cellular Invasion through PEA3 in Lung Cancer

Sunil Upadhyay, Chunyan Liu, Aditi Chatterjee, et al.


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