Repression of BCL2 by the Tumor Suppressor Activity of the Lysyl Oxidase Propeptide Inhibits Transformed Phenotype of Lung and Pancreatic Cancer Cells

Min Wu, Chengyin Min, Xiaobo Wang, Ziyang Yu, Kathrin H. Kirsch, Philip C. Trackman, and Gail E. Sonenshein

1Department of Biochemistry, Boston University School of Medicine; 2Department of Oral Biology, Boston University Goldman School of Dental Medicine; and 3Women's Health Interdisciplinary Research Center, Boston University Medical Campus, Boston, Massachusetts

Abstract

The gene encoding lysyl oxidase (LOX) was identified as the ras reversion gene (rrg), with the ability to revert Ras-mediated transformation of NIH 3T3 fibroblasts. Mutations in RAS genes have been found in ~25% of lung cancers and in 85% of pancreatic cancers. In microarray analysis, these cancers were found to display reduced LOX gene expression. Thus, the ability of the LOX gene to repress the transformed phenotype of these cancer cells was tested. LOX is synthesized as a 50-kDa secreted precursor Pro-LOX that is processed to the 32-kDa active enzyme (LOX) and to an 18-kDa propeptide (LOX-PP). Recently, we mapped the rrg activity of Pro-LOX to the LOX-PP in Ras-transformed NIH 3T3 cells. Ectopic Pro-LOX and LOX-PP expression in H1299 lung cancer cells inhibited growth in soft agar and invasive colony formation in Matrigel and reduced activation of extracellular signal-regulated kinase (ERK) and Akt, with LOX-PP showing substantially higher inhibition of Akt activity in soft agar and ability of these cells to migrate. Nuclear Factor-κB (NF-κB) and its target gene BCL2, which are overexpressed in ~70% to 75% of pancreatic cancers, have recently been implicated in invasive phenotype. LOX-PP substantially reduced NF-κB and Bcl-2 levels. Reintroduction of Bcl-2 into PANC-1 or H1299 cells expressing LOX-PP restored the transformed phenotype, suggesting that Bcl-2 is an essential target. Thus, LOX-PP potently inhibits invasive phenotype of lung and pancreatic cancer cells, suggesting potential therapeutic applications in treatment of these cancers. [Cancer Res 2007;67(13):6278–85]

Introduction

The RAS gene family (HRAS, KRAS, and NRAS) encodes small GTPase proteins that transduce signals that promote growth and survival from membrane-bound receptor tyrosine kinases (reviewed in ref. 1). Oncogenic mutations of RAS, which prevent GTP hydrolysis and cause persistent signaling, promote transformed phenotype. Mutated Ras proteins have been detected in 25% of lung cancers and in up to 85% of pancreatic cancers (2–5), which are two of the most deadly human cancers. The American Cancer Society estimates that these cancers will be responsible for ~190,000 combined deaths in the United States in 2006 (6). Oncogenic RAS activates several distinct signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt and Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase pathways (7). These cascades can activate the Nuclear Factor-κB (NF-κB) family of factors, which directly induces expression of genes that promote cell survival and proliferation, resistance to chemotherapy, neoplastic transformation, and maintenance of invasive phenotype (8–12). Constitutive activation of the RelA NF-κB subunit, which is also known as p65, was found in 70% of pancreatic cancers and plays an important role in pancreatic tumorigenesis (13–15). The antiapoptotic gene Bcl-2 (BCL2) is a tissue-specific NF-κB target gene, and its up-regulation has been implicated in 75% of pancreatic cancers (13, 16). Inhibition of constitutive NF-κB activity by ectopic expression of the specific inhibitory protein IκB-α suppressed the tumorigenicity of the human PANC-1 pancreatic cancer cell line in an orthotopic nude mouse model and reduced BCL2 expression (17). Furthermore, small interfering RNA targeting BCL2 was antiapoptotic and exhibited proapoptotic effects only in tumor cells, while having little effect in fibroblasts or nonmalignant tissues (18), and was shown to delay growth of pancreatic cancer in a xenograft model (18). Of interest, expression of Bcl-2 in prostate cancer was recently shown to promote progression to a metastatic phenotype (19).

The LOX enzyme catalyzes lysine-derived covalent cross-links required for the normal structural integrity of the extracellular matrix (20, 21). The 50-kDa inactive proenzyme (Pro-LOX) is secreted into the extracellular environment and then processed by proteolytic cleavage to a functional 32-kDa LOX enzyme and an 18-kDa propeptide (LOX-PP; ref. 22). The LOX gene was found to have the capacity to revert Ras-mediated transformation of NIH 3T3 cells and was termed the “ras reversion” gene (rrg; refs. 23, 24). In many cancer cell lines examined, LOX gene expression is down-regulated (25–27). In spontaneous revertants or on induced phenotypic reversion, higher, normal levels of LOX are seen (23, 28), and antisense lysyl oxidase causes a return to transformed phenotype (29). Nuclear runoff transcription assays showed a marked reduction in synthesis of LOX RNA resulting from Ras transformation. Potential mechanisms for the down-regulation of LOX gene expression include inactivation by methylation and loss of heterozygosity (29).

We have shown that ectopic expression of the precursor Pro-LOX in Ras-transformed NIH 3T3 cells inhibited the activities of Akt and ERK kinases and downstream induction of p50/p65 NF-κB (30). Subsequently, the LOX rrg activity was mapped to the
propeptide domain of LOX and not the LOX enzyme (31). LOX-PP reduced Ras-dependent transformation of NIH 3T3 cells as determined by effects on proliferation, growth in soft agar, and the inhibition of the PI3K/PDK1/Akt pathway and NF-κB activity (31). In these studies, we have tested whether the LOX-PP rgg activity functions in human lung and pancreatic cancer cells that carry NRAS and KRAS mutations, respectively (and mutant P53 genes). The results show that LOX-PP strongly inhibits Ras signaling and NF-κB activity. Furthermore, we identify Bcl-2 as an essential target of LOX-PP–mediated inhibition of transformed phenotype.

Materials and Methods

Cell culture and treatment conditions. H1299 lung cancer and PANC-1 pancreatic cancer cell lines were kindly provided by Zhi-Xiong Jim Xiao (Boston University School of Medicine, Boston, MA) and Edward E. Whang (Brigham and Women's Hospital, Boston, MA), respectively. Cells were grown as described by the American Type Culture Collection. Where indicated, cultures of stable H1299 or PANC-1 transfectants/infectants were treated with 2 μg/mL doxycycline (Sigma) to induce transient expression.

DNA constructs and transfection and infection analyses. The cDNA fragments encoding murine Pro-LOX and LOX-PP containing a COOH-terminal V5/His tag were cloned into the retroviral vector pC4 bsrR(TO) containing a doxycycline-inducible promoter or vector pCXneoTR2. An optimized ratio of 1:10 (10 ng of NF-κB luciferase reporter DNA and 100 ng of LOX-PP) was used for the transfection. pCMV Bcl-2 Cells were selected with 10 μg/mL blasticidin (Invitrogen) and 600 μg/mL geneticin (Sigma). Single clones of cells expressing LOX-PP were isolated by limiting dilution. Because of the low transfection efficiency of PANC-1 cells, stable lines were prepared by retroviral infection. Retrovirus stocks were generated by cotransfecting 293T cells with amphotropic (pCL-Ampho) vector (Imgenex) and either EV pC4bsrR(TO) or pC4 bsrR(TO)-LOX-PP. 293T cells were also transfected with the regulator plasmid pCXneoTR2. After 48 h, PANC-1 cells were dually infected with filtered culture supernatant from the 293T cells containing viruses that carry the regulator vector pCXneoTR2 and effector vector pC4 bsrR(TO)-LOX-PP or empty pC4 bsrR(TO) and supplemented with 6 μg/mL polybrene. Infected cells were selected with 10 μg/mL blasticidin and 1.2 mg/mL geneticin to generate pools of stable infectants of LOX-PP and EV control cells, and single clones were isolated by limiting dilution.

pBABE-Bcl-2 expression vector and pBABE-LOX–PLV Env were kindly provided by Stanley Korsmeyer (Dana-Farber Cancer Institute, Boston, MA; ref. 33). Bcl-2 stable lines were established by transfection of pBABE-Bcl-2 expression vector and pBABE-LOX–PLV Env DNA into PANC-1-LOX-PP and H1299-LOX-PP clones and H1299-EV lines and selection with 5 μg/mL puromycin. For the NF-κB transactivation assay, a six-copy NF-κB element-driven luciferase reporter construct (kindly provided by G. Rawadi, Hoechst Marion Roussel, Romainville, France) was used. CMV-driven LOX-PP construct or EV DNA was used to transiently transfect PANC-1 cells using LipofectAMINE 2000. An optimized ratio of 1:10 (10 ng of NF-κB luciferase reporter DNA and 100 ng of LOX-PP) was used for the transfection. pBLLucilla DNA (10 ng; Promega) was cotransfected to normalize for transfection efficiency. Seventy-two hours after transfection, a dual luciferase assay was done according to the manufacturer’s instruction (Promega).

Immunoblot analysis. To isolate nuclear proteins, washed cells were lysed by suspension in ice-cold lysis buffer [10 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 2 mmol/L sodium orthovanadate (Sigma), 1 mmol/L PMSF, and 10 μg/mL each of aprotinin, leupeptin, pepstatin, and peptastatin (Sigma)] and centrifuged at 14,000 × g for 30 min at 4°C. The supernatants were stored at −80°C.

Figure 1. LOX expression is decreased in lung and pancreatic primary tumors and cell lines. A, LOX mRNA expression analysis in primary lung cancer samples. Box plots of data from the Garber lung microarray data set (reporter number IMAGE:341680) were accessed using the Oncomine Cancer Profiling Database (http://www.oncomine.org) and are plotted on a log scale (http://www.pnas.org/cgi/content/full/241500798/DC1). The data set includes 6 normal lung samples (Normal) and 40 lung adenocarcinoma patient samples (Tumor). Line within the box, median expression value for each group. Top edge of the box, 75th percentile of the distribution; bottom edge, 25th percentile. The lines (whiskers) emerging from each box extend to the smallest and largest observations; for normal lung tissues, the line is at the top of the box. Black dots outside the ends of the whiskers, outlier data points. A Student’s t test, done directly through the Oncomine 3.0 software, showed that the difference in LOX expression between the two groups was significant (P = 0.01). B, LOX mRNA expression analyses from microarray data on lung cancer cell lines (GEO accession number: GDS89). C, data analysis was done on LOX mRNA expression in 8 primary pancreatic tumor samples (Δ) and 13 pancreatic cancer cell lines (♀) in the Stanford microarray database (http://genome-www5.stanford.edu).
10 mmol/L KCl, 5 mmol/L MgCl₂, plus DTT, 1% NP-40, and protease inhibitor cocktail (Roche Diagnostics) for 15 min. Lysis was verified by crystal violet staining. The nuclei were pelleted by centrifugation for 4 min at 2,500 rpm at 4°C, and the supernatant was discarded. The nuclear pellets were washed once in lysis buffer without detergent, and proteins were extracted in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% sodium lauryl sarcosine, 0.1% SDS, 1 mmol/L EDTA plus DTT, and protease inhibitor cocktail as above. The extracts were briefly sonicated, followed by centrifugation for 30 min at 14,000 × g at 4°C, and the supernatant containing the nuclear proteins was removed. For preparation of whole-cell protein lysates, cells were incubated in lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100] plus protease inhibitor cocktail and phosphatase inhibitors (20 mmol/L NaF, 10 mmol/L Na₃VO₄, and 1 mmol/L Na₂VO₄). Protein concentrations were determined using the detergent-compatible protein assay kit (Bio-Rad). Samples (50 μg) were subjected to immunoblotting as described (30). The antibody reagents for Akt, phosphorylated Akt (Ser473), phosphorylated ERK1/2 (Thr202Tyr204), and ERK1/2 were obtained from Cell Signaling. Antibodies against β-actin and V5 epitope were from Sigma and Invitrogen, respectively. Antibodies against p65, It-B-α, and Bcl-2 were from Santa Cruz Biotechnology. To detect expression of recombinant proteins in cell culture medium, 1 or 2 mL of 10 mL culture medium were subjected to immunoprecipitation using a V5 antibody (Sigma) and protein A-Sepharose (Invitrogen). Immunoblot analysis was done using anti-V5 antibody followed by incubation with protein A conjugated to horseradish peroxidase (HRP) or goat anti-mouse IgG conjugated to HRP specific for the Fc fragment.

**Soft agar growth assay.** Stable cell lines or clones were plated at 5,000 cells per well in 0.35% top agarose (SeaPlaque Agarose, FMC BioProducts) with a base agarose of 0.7% agarose supplemented with complete medium. Cultures were treated with 2 μg/mL doxycycline and incubated in a humidified incubator at 37°C for 2 weeks. Cells were stained with 0.5 mL of 0.0005% crystal violet, and colonies were counted visually. All experiments were done in triplicate with two independent experiments.

**Matrigel outgrowth assay.** Matrigel (BD Biosciences) was diluted to a concentration of 6.3 mg/mL with serum-free medium (DMEM) and stored at −80°C. Matrigel was thawed on ice overnight. For the bottom layer, 200 μL of Matrigel solution were added into a 24-well tissue culture plate and incubated at 37°C for 30 min to allow the Matrigel to solidify. A single-cell suspension (2.5 × 10⁵ cells/mL) in serum-free medium (DMEM) was made by passing the cell suspension through a 21-gauge needle five times. Ten microliters (2,500 cells) were mixed with 190 μL Matrigel and plated, in duplicate, onto the solidified bottom layer. The plates were incubated at 37°C for 30 min to allow the Matrigel to solidify, and complete medium with 2 μg/mL doxycycline was then added. Following incubation at 37°C for 5 to 7 days, cell growth was analyzed using a Zeiss Axiovert 200 M microscope. Experiments were done twice with similar results.

**Migration assay.** Suspensions of 1 × 10⁵ cells, which had been pretreated with doxycycline for 24 h, were placed in the upper compartments of Transwells (Costar) on an 8-mm-diameter polycarbonate filter and incubated at 37°C for 6 h. Migration of the cells to the lower side of the filter was evaluated with the acid phosphatase enzymatic assay using p-nitrophenyl phosphate (Sigma) and A₄₁₀nm determination.

**Reverse transcription-PCR analysis.** Total RNA was extracted and purified using Trizol reagent (Invitrogen). RNA samples (5 μg) were reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with random primers. Amplification of the BCL2 cDNA was done with the following primers: 5'-CATTTCCACGTCAACAGAATTG-3' (forward) and 5'-AGCACAGGATTGGATATTCCAT-3' (reverse). The primers used for the

**Figure 2.** Pro-LOX and LOX-PP expression inhibits RAS-mediated transformation of H1299 lung cancer cells. A, H1299-EV, H1299-Pro-LOX2, and H1299-Pro-LOX4 cells were incubated in the presence of 2 μg/mL doxycycline for 24 h, and whole-cell protein extracts (30 μg) were subjected to immunoblot analysis with a V5 monoclonal antibody. The three panels are from the same gel. The positions of molecular mass markers are indicated. B, the culture medium of H1299-LOX-PP4 and H1299-LOX-PP7 cells was collected 24 h after incubation in the absence (−) or presence (+) of doxycycline (DOX). Samples (1 mL) were subjected to immunoprecipitation with a V5 antibody followed by immunoblot analysis using anti-V5 antibody, and detection of LOX-PP was done using protein A-conjugated HRP. C, growth in soft agar assays. Cells of the stable lines or clones were plated, in triplicate, in 0.4% soft agar in the presence of doxycycline. After 2 wks, the colonies were stained with 0.0005% crystal violet and photographed using a digital camera coupled to a Nikon Diaphot-TMD inverted microscope. D, Matrigel outgrowth assays. After induction with doxycycline for 24 h, the indicated cells were subjected to Matrigel outgrowth assays in the presence of doxycycline for 1 wk. Cultures were photographed using a Zeiss Axiovert 200 M microscope and an Orca ER camera at ×100 magnification, and representative images are shown from one of two independent experiments with similar results.
amplification of the control GAPDH are 5′-TCACCATCTCCAGGAG-3′ (forward) and 5′-GCCATCCACTCTGTTG-3′ (reverse). PCR was done in a thermal cycler for 25 cycles for BCL2 and 16 cycles for GAPDH as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Results

LOX gene expression is reduced in lung and pancreatic cancers. To determine whether LOX gene expression is reduced in lung cancer, we analyzed the microarray data of 40 primary lung adenocarcinoma samples available on the Oncomine Web site3 versus six normal lung tissue samples. As seen in the resulting box plot, the levels of LOX mRNA were substantially lower in primary tumor samples versus the normal tissues (Fig. 1A). A Student’s t test, done directly through the Oncomine 3.0 software, showed that the difference in LOX expression between the two groups was significant (P = 0.01). Additional microarray data on nine lung cancer cell lines revealed similar findings; all but one have low LOX mRNA levels (GEO accession number: GDS89; Fig. 1B). Similarly, analysis of data on 8 primary pancreatic tumor samples and 13 pancreatic cancer cell lines in the Stanford Microarray database4 showed that all have low levels of LOX mRNA expression (Fig. 1C).

LOX-PP inhibits anchorage-independent growth and invasive colony formation by H1299 lung cancer cells. To investigate whether overexpression of Pro-LOX or LOX-PP will inhibit the transformed phenotype of H1299 lung cancer cells that carry an NRAS mutation, we used a doxycycline-inducible system. H1299 cells were cotransfected with vectors that expressed either the full-length proenzyme Pro-LOX or the propeptide portion of the precursor (LOX-PP), with COOH-terminal V5 tags or with empty parental retroviral vector pC4bsrR(TO) (EV) DNA and the regulator vector pCXneoTR2. Stable lines were derived under dual antibiotic selection, and single clones for Pro-LOX (H1299-Pro-LOX2 and Pro-LOX4) and LOX-PP (H1299-LOX-PP4 and LOX-PP7) were isolated to reduce problems with leaky expression. To verify ectopic expression of the transgene, whole-cell protein extracts and the culture medium were isolated 24 h after induction with 2 μg/mL doxycycline. Ectopic expression of Pro-LOX was detected in the extracts of the H1299-Pro-LOX2 and Pro-LOX4 cells by immunoblotting with a V5 monoclonal antibody (Fig. 2A). The Pro-LOX (50 kDa) was efficiently secreted into the medium and processed to the 32-kDa mature LOX enzyme and the propeptide, which is not detected due to the loss of the tag. Expression of LOX-PP by the H1299-LOX-PP4 and LOX-PP7 cells, which is efficiently secreted, was analyzed in the culture medium by immunoprecipitation with a V5 antibody followed by immunoblot analysis (Fig. 2B). The 18-kDa LOX-PP was detected as a band of >25 kDa, presumably due to N-glycosylation, as seen previously (32, 34).

Having established inducible expression in the H1299 cells, the effects of Pro-LOX and LOX-PP on growth of H1299 cells in soft agar, a hallmark of transformed phenotype, were evaluated. Growth of H1299 cells in soft agar was robust (Fig. 2C). Cells expressing the EV DNA formed large colonies within 14 days of culture. Ectopic expression of Pro-LOX or of LOX-PP substantially reduced the ability of H1299 cells to grow in soft agar; only small colonies were observed with either H1299-Pro-LOX2 and Pro-LOX4 or H1299-LOX-PP4 and LOX-PP7 cells (Fig. 2C). Many highly malignant cells form colonies with an invasive branching appearance when plated

1 http://www.oncomine.org
2 http://genome-www5.Stanford.edu

Figure 3. LOX-PP inhibits RAS-mediated signaling in H1299 lung and anchorage-independent growth of PANC-1 pancreatic cancer cells. A and B, H1299-EV, H1299-Pro-LOX2, H1299-Pro-LOX4, H1299-LOX-PP4, and H1299-LOX-PP7 cells were cultured in DMEM + 10% FBS in the presence of doxycycline for 24 h. Whole-cell extracts were prepared and samples (50 μg protein) were subjected to immunoblotting using antibodies against Ser106-phosphorylated Akt (p-Akt) and total Akt (A) and phosphorylated Thr37/46-activated ERK1/2 (p-ERK1/2) and total ERK1/2 (B). C and D, PANC-1 cells were infected with retroviral constructs and cells stably integrated with empty parental control vector (EV) and LOX-PP DNA were obtained. Two clonal derivatives were isolated from the PANC-1-LOX-PP cells and these were called PANC-1-LOX-PP7 and PANC-1-LOX-PP8. C, PANC-1-EV, PANC-1-LOX-PP, PANC-1-LOX-PP7, and PANC-1-LOX-PP8 cells were incubated in the presence of doxycycline for 24 h. Samples of cell culture medium (2 mL) were subjected to immunoprecipitation overnight with a V5 antibody followed by further incubation with protein A-Sepharose for 2 h. Immunoprecipitated LOX-PP proteins were identified by immunoblot analysis using anti-V5 antibody followed by incubation with goat anti-mouse IgG conjugated to HRP specific for the Fc fragment. D, the PANC-1-EV and PANC-1-LOX-PP cells and the clonal derivatives PANC-1-LOX-PP7 and PANC-1-LOX-PP8 were plated, in triplicate, in 0.4% soft agar in the presence of doxycycline. Top, after 2 wks, the colonies were stained and photographed as above; bottom, colony numbers were determined by counting colonies from three random fields. Columns, percentage colony formation compared with EV cells; bars, SD.
in Matrigel. The H1299-EV, H1299-Pro-LOX, and H1299-LOX-PP cells were seeded in Matrigel and grown in the presence of doxycycline. After 7 days, H1299 EV cells readily formed branching outgrowth structures in Matrigel (Fig. 2D). Overexpression of Pro-LOX or LOX-PP potently abolished this feature of mesenchymal cells, consistent with the activity residing within the LOX-PP domain (Fig. 2D). Expression of mature 32-kDa LOX, without the propeptide region, was unable to inhibit either growth in soft agar or branching colony formation in Matrigel (data not shown). Thus, LOX-PP reduces anchorage-independent growth and invasive colony formation of these lung cancer cells.

LOX-PP inhibits RAS signaling pathways in lung cancer cells. The Akt and ERK kinases are two major mediators of RAS signaling. To examine whether expression of Pro-LOX and LOX-PP affects activation of Akt and ERK in H1299 cells, immunoblotting for the active form of Akt and ERK proteins was done using phospho-specific antibodies. Cells were treated with doxycycline for 24 h and whole-cell protein extracts were analyzed for levels of phospho-specific Akt and ERK. Overexpression of either Pro-LOX or LOX-PP potently reduced activation of Akt and ERK (Fig. 3A and B). Antibodies against Akt and ERK confirmed essentially equal protein loading. Thus, Pro-LOX and LOX-PP potently inhibit RAS signaling via the Akt and ERK pathways. Furthermore, rrg activity resides within the propeptide domain and was selected for experiments with the pancreatic cancer cells.

LOX-PP reduces RAS-mediated transformation of pancreatic cancer cells. Given that LOX gene expression is consistently reduced in pancreatic cancers (Fig. 1C), which are frequently driven by RAS mutations, we next evaluated the effects of LOX-PP expression on Panc-1 cells, which carry a KRAS mutation and express low levels of the LOX gene product.

Figure 4. LOX-PP inhibits RAS-mediated transformation of Panc-1 cells. A to C, Panc-1-EV, Panc-1-LOX-PP7, and Panc-1-LOX-PP8 cells were treated with doxycycline for 24 h. A, for migration assays, suspensions of 1 × 10⁶ cells were placed in the upper compartments of Transwells and incubated at 37°C for 6 h. Migration of the cells to the lower side of the filter was measured with the acid phosphatase enzymatic assay. B, whole-cell protein extracts (50 μg) were subjected to immunoblot analysis using antibodies against Ser⁴⁷³-phosphorylated Akt, phosphorylated Thr²⁰²Tyr²⁰⁴ ERK1/2, or β-actin, which confirmed equal protein loading. C, nuclear or whole-cell extracts or RNA were prepared. Samples of nuclear extracts (30 μg) and whole-cell protein extracts (50 μg) were subjected to immunoblotting analysis against NF-κB, IκBα, and β-actin, respectively. Alternatively, whole-cell protein extracts (50 μg) were subjected to immunoblot analysis for Bcl-2 expression. Immunoblotting for β-actin confirmed equal protein loading. RNA was subjected to RT-PCR for Bcl2 and GAPDH as loading control. D, Panc-1 cells were transiently transfected, in triplicate, with 100 ng of CMV-driven LOX-PP construct or control EV DNA, 10 ng of NF-κB element-driven luciferase reporter construct, and 10 ng of Renilla luciferase pRL-TK vector to normalize for transfection efficiencies. After 72 h, dual luciferase assays were conducted, and NF-κB–driven luciferase activity was normalized to the Renilla activity in the same sample. Columns, values for the LOX-PP cells presented relative to the EV control set at 100%; bars, SD.
PANC-1-LOX-PP7, PANC-1-LOX-PP8, and PANC-1-EV cells stably transfected with pBABE-Bcl-2 (Bcl-2), the product of a pancreatic cancers (13, 16). Thus, the effects of LOX-PP on Bcl-2 activity of NF-κB indicate that ectopic LOX-PP potently inhibits both expression and correlated with the decrease in p65 levels. Together, these data (Fig. 4A). Concomitantly, there was an induction in the inhibitory protein IκBα as well as on its subunit and Bcl-2. LOX-PP expression of the p65 NF-κB subunit is implicated in approximately 70% of pancreatic cancers and serves as a downstream effector for the Akt and ERK pathways (13, 14, 35), we examined the effects of LOX-PP expression on the p65 NF-κB subunit as well as on its inhibitory protein IκBα. Nuclear protein extracts or whole-cell protein extracts were prepared separately from the same cell sample that had been treated with doxycycline for 24 h and subjected to immunoblot analysis. A substantial reduction in nuclear levels of p65 was observed in response to overexpression of LOX-PP (Fig. 4C). Concomitantly, there was an induction in the IκBα protein levels in the whole-cell protein extracts (Fig. 4C). Furthermore, ectopic expression of LOX-PP in PANC-1 cells reduced NF-κB element-driven promoter activity by approximately 73% (Fig. 4D), which correlated with the decrease in p65 levels. Together, these data indicate that ectopic LOX-PP potently inhibits both expression and activity of NF-κB, an important mediator of RAS signaling.

**Bcl-2 is an essential target for LOX-PP signaling in pancreatic and lung cancer cells.** Bcl-2, the product of a tissue-specific NF-κB target gene, is elevated in approximately 75% of pancreatic cancers (15, 16). Thus, the effects of LOX-PP on Bcl-2 expression were examined. LOX-PP potently reduced levels of Bcl-2 protein and BCL2 RNA as judged by immunoblotting and reverse transcription-PCR (RT-PCR) analyses, respectively (Fig. 4C). Recent studies have implicated Bcl-2 as playing a role in promoting transformed phenotype as well as cell survival (36–38). To investigate whether reintroduction of Bcl-2 into LOX-PP cells would overcome LOX-PP rgg activity, the Bcl-2 expression vector pBABE-Bcl-2 and control pBABE-EV plasmid DNAs were transfected into the PANC-1-LOX-PP clones and control PANC-1-EV cells. Stable transfectants were selected and overexpression of Bcl-2 was confirmed (Fig. 5A). Ectopic Bcl-2 expression significantly restored the ability of PANC-1-LOX-PP cells to grow in soft agar (Fig. 5B) and enhanced their ability to migrate (Fig. 5C). Ectopic Bcl-2 expression did not seem to affect cells with EV in terms of either the number of colonies formed in soft agar or the ability of the cells to migrate (Fig. 5B and C).

To determine whether the effects on Bcl-2 could be extended to lung cancer cells, we first tested the H1299-EV control cells and H1299-LOX-PP4 and LOX-PP7 clones for effects of LOX-PP on expression of the p65 NF-κB subunit and Bcl-2. LOX-PP expression in H1299 cells similarly caused a reduction in these two proteins (Fig. 6A). We then asked whether ectopic expression of Bcl-2 restored the ability of these cells to form invasive colonies in Matrigel (Fig. 6B). An essentially complete reversion of invasive colony formation was seen upon ectopic Bcl-2 expression (Fig. 6C). Thus, Bcl-2 is an essential target for the antitransforming ability of LOX-PP.

**Figure 5.** Bcl-2 promotes transformed phenotype and is an essential target of LOX-PP signaling in PANC-1 cells. A, PANC-1-EV, PANC-1-LOX-PP7, and PANC-1-LOX-PP8 clones were transfected with either pBABE-Bcl-2 (+, Bcl-2) or pBABE-EV (−, Bcl-2) vector DNAs, and stable transfectants were obtained using puromycin selection. Samples of whole-cell protein extracts (50 μg) were subjected to immunoblotting for Bcl-2 expression, and β-actin as loading control. B, top, PANC-1-LOX-PP7, PANC-1-LOX-PP8, and PANC-1-EV cells stably transfected with pBABE-Bcl-2 (Bcl-2) or pBABE-EV were grown in soft agar, stained, and photographed as in the legend of Fig. 3. Bottom, colony numbers were determined by counting three random fields. Columns, percentage colony formation compared with PANC-1-EV cells transfected with pBABE-EV set at 100%; bars, SD. C, PANC-1-LOX-PP7, PANC-1-LOX-PP8, and PANC-1-EV cells stably transfected with pBABE-Bcl-2 (Bcl-2) or pBABE-EV (pBABE) were treated with doxycycline for 24 h and subjected to migration assay as in the legend of Fig. 4A.
Discussion

Here, we show, for the first time, the ability of the propeptide of lysyl oxidase to overcome RAS signaling and transformation of lung and pancreatic cancer cells that harbor RAS mutations and identify Bcl-2 as an essential downstream target of LOX-PP. In H1299 cells, which carry an NRAS mutation, LOX-PP potently inhibited activation of Akt and ERK, two of the major effectors of RAS signaling, growth in soft agar, and invasive colony formation in Matrigel. Similarly, LOX-PP decreased RAS-induced signaling and the transformed phenotype of PAN-1 pancreatic cancer cells, which carry a KRAS mutation, as judged by Akt and ERK activity, decreased ability to grow in soft agar or to migrate. In both cells, LOX-PP reduced levels of p65 NF-κB and Bcl-2. Recently, we showed that ectopic expression of LOX-PP effectively inhibited Her-2/neu signaling, an upstream activator of Ras in mouse breast carcinoma cells (32). It is also noteworthy that both H1299 and PAN-1 cells are p53 deficient. More than 50% of all human cancers bear P53 mutations, and this deficiency renders them greatly resistant to different means of therapy, including chemotherapy and irradiation (39-42). Thus, the LOX-PP ability to overcome RAS-mediated transformation in the absence of a functional p53 and in the presence of a mutant Ras protein suggests further investigation of the therapeutic potential of LOX-PP is warranted.

Aberrant activation of NF-κB factors typifies many primary cancers. Constitutive activation of the p65 NF-κB subunit was found in 70% of human pancreatic adenocarcinomas and human pancreatic cancer cell lines but not in immortalized, nontumorigenic pancreatic epithelial cells or normal pancreatic tissue (13, 14, 35, 43). Evidence indicates that the aberrant activation of NF-κB contributes to pancreatic tumorigenesis (15, 35, 43, 44). As a multifaceted regulator, NF-κB controls a network of important proteins that increase metastasis and protect cancer cells from apoptosis (8, 9, 12, 45). Importantly, many of NF-κB–induced genes are important drug targets in cancer therapeutic strategies (12), including Bcl-2. Interestingly, LOX-PP caused comparable decreases in p65 protein levels in whole-cell and nuclear extract (data not shown). This implies that the NF-κB regulation mediated by LOX-PP is not simply due to the sequestration in the cytoplasm as seen conventionally.

Here, we identify Bcl-2 as an essential target for LOX-PP antitransformation signaling. LOX-PP potently down-regulated Bcl-2 protein in PAN-1 and H1299 cells. Recent studies have linked Bcl-2 to invasive properties and metastasis of tumors (36, 37). Expression of Bcl-2 in breast cancer cells strikingly increased their ability to metastasize to the lung upon injection into nude mice (46). Our data show that overexpression of Bcl-2 potently promotes cell migration, growth in soft agar of PAN-1-LOX-PP cells, and invasive phenotype of H1299-LOX-PP clones. Importantly, BCL2-mediated transformation occurred only in LOX-PP cells, but not in EV cells, implying that Bcl-2 is an essential target of LOX-PP, and resumption of Bcl-2 expression is capable of overcoming the rgg activity of LOX-PP. Two major mechanisms regulate Bcl-2 levels: (a) control of BCL2 gene transcription, mediated by several transcription factors, including NF-κB, and (b) ubiquitin-dependent degradation mediated by MAPK. Although changes in BCL2 mRNA levels were observed, to date, we have been unable to find evidence for regulation of Bcl-2 protein stability. LOX-PP failed to alter ubiquitination of Bcl-2 when a hemagglutinin (HA)-tagged ubiquitin was expressed and immunoprecipitation with HA antibody was followed by immunoblot analysis for Bcl-2 (data not shown). These results suggest that LOX-PP–mediated signaling events may exert control of BCL2 gene transcription. Lastly, addition of the general caspase inhibitor Z-VAD-fmk had only modest effects on colony formation by either H1299 or PAN-1 cells following induction of LOX-PP (data not shown), indicating the propeptide does not induce significant levels of anoikis. In summary, LOX-PP significantly inhibits transformed phenotype of both pancreatic and lung cancer cells that bear mutant RAS and P53 genes. Thus, LOX-PP may be useful in novel therapeutic strategies for cancer treatment.

Acknowledgments


Grant support: American Cancer Society grant IRG-72-001-30-IBG, NIH grant CA82742, and Department of the Army grant DAMD 05-1-0286.

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We thank Edward E. Whang and Zhi-Xiong Jim Xiao for cell lines and Stanley Korsmeyer, Tsuyoshi Akagi, and Georges Rawadi for plasmid and retroviral vectors.
References


Repression of BCL2 by the Tumor Suppressor Activity of the Lysyl Oxidase Propeptide Inhibits Transformed Phenotype of Lung and Pancreatic Cancer Cells

Min Wu, Chengyin Min, Xiaobo Wang, et al.