The Propeptide Domain of Lysyl Oxidase Induces Phenotypic Reversion of Ras-transformed Cells*

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Lysyl oxidase is an extracellular enzyme critical for the normal biosynthesis of collagens and elastin. In addition, lysyl oxidase reverts ras-mediated transformation, and lysyl oxidase expression is down-regulated in human cancers. Since suramin inhibits growth factor signaling pathways and induces lysyl oxidase in ras-transformed NIH3T3 cells (RS485 cells), we sought to investigate the effects of suramin on the phenotype of transformed cells and the role of lysyl oxidase in mediating these effects. Suramin treatment resulted in a more normal phenotype as judged by growth rate, cell cycle parameters, and morphology. β-aminopropionitrile, the selective inhibitor of lysyl oxidase enzyme activity, was remarkably unable to block suramin-induced reversion. By contrast, ectopic antisense lysyl oxidase demonstrated that lysyl oxidase gene expression mediated phenotypic reversion. Since lysyl oxidase is synthesized as a 50 kDa precursor and processed to a 30 kDa active enzyme and 18 kDa propeptide, the effects of these two products on the transformed phenotype of RS485 cells were then directly assessed in the absence of suramin. Here we report, for the first time, that the lysyl oxidase propeptide, and not the lysyl oxidase enzyme, inhibits ras-dependent transformation as determined by effects on cell proliferation assays, growth in soft agar, and Akt-dependent induction of NF-κB activity. Thus, the lysyl oxidase propeptide, which is released during extracellular proteolytic processing of pro-lysyl oxidase functions to inhibit ras-dependent cell transformation.

Lysyl oxidase catalyzes oxidative deamination of peptidyl lysine and hydroxylysine residues in collagen, and peptidyl lysine residues in elastin. The resulting peptidyl aldehydes spontaneously condense and undergo oxidation reactions to form the lysine-derived covalent cross-links required for the normal structural integrity of the extracellular matrix (1–3). Lysyl oxidase is synthesized as a 48–50 kDa proenzyme, secreted into the extracellular environment where it is then processed by proteolytic cleavage to a functional 30 kDa enzyme and an 18 kDa propeptide (4). Evidence supports that 30 kDa lysyl oxidase is active whereas the 50 kDa proenzyme is enzymatically inactive (5–7). Procollagen C-proteinases are active in processing pro-lysyl oxidase and are products of the Bmp1 gene and the structurally related Tll1 and Tll2 genes (6–8).

Lysyl oxidase gene expression was found to inhibit the transforming activity of ras and was hence named the “ras rejection gene” (trg) (9, 10). Lysyl oxidase is down-regulated in ras-transformed cells and in many cancer cell lines. Reduced lysyl oxidase levels are also observed in human cancers (9, 11–15), whereas in spontaneous revertants or upon induced phenotypic reversion higher normal levels of lysyl oxidase are again seen (9, 14). Conversely stable phenotypic revertants of ras-transformed NIH3T3 cells return to a transformed phenotype upon transfection with an antisense lysyl oxidase vector (9, 10, 16). Antisense lysyl oxidase transfection triggers transformation of normal rat kidney fibroblasts (17). Thus, the lysyl oxidase gene has tumor suppressor activity. Recently, we showed that ectopic expression of pro-lysyl oxidase in ras-transformed cells inhibits the activities of the phosphatidylinositol 3-kinase (PI3K),† Akt, and MEK kinases that lead to the activation of NF-κB (18).

Suramin is a polysulfonated naphthylurea, initially used in the treatment of trypanosomiasis and onchocerciasis (19). Its anticancer activity was later identified, and suramin has been introduced into clinical trials for various forms of cancer (20–24). Suramin interrupts autocrine growth factor pathways by inhibiting the binding of growth factors to their receptors (19, 25–28). We have recently shown that lysyl oxidase is dramatically up-regulated by suramin in c-Ha-ras-transformed NIH3T3 cells (RS485 cells) because of its inhibition of an FGF-2-mediated autocrine pathway (29). The question is now raised whether suramin causes phenotypic reversion of RS485 cells, and whether this reversion depends on lysyl oxidase expression or activity. In addition, the role of lysyl oxidase enzyme activity in mediating phenotypic reversion was investigated. The results indicate that suramin-induced phenotypic reversion requires lysyl oxidase expression as expected, but reversion surprisingly does not require lysyl oxidase enzyme activity. Data show that reversion is mediated instead by the lysyl oxidase propeptide. The ability of the lysyl oxidase propeptide, and not active lysyl oxidase, to stimulate phenotypic reversion was confirmed in studies of ras-transformed cells performed in the

†The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein; β-Gal, β-galactosidase; BAPN, β-aminopropionitrile; PDK1, phosphoinositide-dependent kinase-1.

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Lysyl Oxidase Induces Phenotypic Reversion

Lysyl Oxidase Induces Phenotypic Reversion—RS485 cells were plated in 6-well plates at a density of 35,000 cells/well and were grown in complete medium containing 10% FBS, as described above. Additions of suramin or β-aminopropionitrile (BAPN), when appropriate, were initiated 24 h after plating. Media were changed every 3 days in the continuous presence of suramin or BAPN, as indicated for each experimental design in the “Results.” Cell density was determined in triplicate every day by crystal violet staining, as described (32, 33). Cells were fixed with 10% formalin in PBS, washed with PBS, and then stained for 30 min with 0.1% crystal violet at room temperature with shaking. Unbound dye was then removed by washing with water until washes were colorless. Bound dye was then eluted with 10% acetic acid, and quantitated by measuring the absorbance at 590 nm. For quantitative analyses of growth rates, the logarithmic value of absorbance versus time was plotted ± S.E. and the rates were calculated by linear regression analyses. In addition, data were plotted as total absorbance ± S.E. versus time. Experiments were performed three times each with consistent findings.

Lysyl Oxidase Enzyme Activity—PR4 cells were plated in 100-mm cell culture plates and then grown and re-fed every 2 days as described above in the constant presence of 0, 200, and 400 μM BAPN for 7 days until visually confluent. Cells were then re-fed with serum-free medium supplemented with 0.1% bovine serum albumin still in the constant presence or absence of BAPN. After 24 h conditioned 0.3-ml aliquots of media samples were assayed in quadruplicate using a tritiated recombinant human tropoelastin as substrate as previously described (4). Incubations were performed at 37 °C for 18 h (34), and data were expressed as total cpm released ± S.E. per culture.

Cell Cycle Analysis—RS485, NIH3T3, and PR4 cells were plated on 100-mm plates and were grown until confluent with 0 or 150 μM suramin. Cell density was determined in triplicate every day by flow cytometry using a FACScan flow cytometry with CELLQUEST acquisition and analysis software (BD Biosciences). Two days after seeding, triplicate samples of denatured RNA were electrophoresed on a 1% agarose gel stained with ethidium bromide. Bands were quantitated by densitometric scanning. Northern analysis as described above. In selected experiments, mature 30 kDa lysyl oxidase (36) was dialyzed against 16 mM potassium phosphate buffer, pH 7.8 for 5 h, and 4 μg coated in 24-well plates at the same time as described for the propeptide.

Focus Formation Assay in Soft Agar—RS485 cells and Myc-transformed M158 cells were plated, in duplicate, at 104 cells/ml in Ham’s F-12 nutrient mixture medium containing 10% FBS and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine). In the presence of 2.5 μg of purified bovine aorta lysyl oxidase enzyme (30 kDa form) (41), or with 2.5 μg recombinant rat lysyl oxidase propeptide (18 kDa form) (40), or the same volume of vehicle phosphate buffer, pH 7.8. After 2 weeks of incubation in a humidified incubator at 37 °C, the colonies were stained with 0.5 ml of 0.0005% crystal violet and photographed using a digital camera coupled to a dissection microscope (×50 magnification). Three random fields were counted from each of two duplicate samples, and average values presented ± S.D.

Transient Transfections, Luciferase Assays, and Fluorescence Microscopy—RS485, NIH3T3 and PR4 cells were plated in 35-mm culture dishes. Cells were transfected overnight, in triplicate, with the indicated expression vectors by using FuGENE 6 transfection reagent (Roche Applied Science) in Dulbecco’s modified Eagle’s medium containing 0.5% FBS. The plasmids used were pCMVneo-Myr-Akt kindly provided by Z. Luo (Boston University Medical School, Boston, Mass.), NF-κB-luciferase which was a gift from G. Rawadi (Hoechst-Marion-Roussel, Romainville, France), pEGFP-C1-PDK1 kindly provided by J. Chung (Korean Advanced Institute of Science and Technology, Taejon, Republic of Korea), and pcDNA3.1 (+) VLOPP propeptide (Lysyl Oxidase Induces Phenotypic Reversion) and pcDNA4-LO enzyme expression vectors. The expression vector for the lysyl oxidase propeptide pcDNA3.1 (+) VLOPP was generated from pSV40-β-Gal (35) by digestion with BamH I and EcoRI and gel-purified and cloned into pcDNA3.1 (+) (Invitrogen), resulting in pcDNA3.1 (+) VLOPP. This construct contains the rat cDNA sequence (−94 to +486) that includes a portion of the 5′-UTR, the signal peptide, the entire rat lysyl oxidase coding region and no mature lysyl oxidase sequence. The insert was directly confirmed by DNA sequencing. The expression vector for mature lysyl oxidase was accomplished by excision of nucleotides encoding amino acid residues 23–157 from a construct of murine lysyl oxidase cDNA to 33–125, and then cloned into pcDNA4 as previously reported (42). The plasmid pCMVneo-Myr-Akt (Promega kit) was transfected in NIH3T3 cells with a ratio of 1:1 for luciferase reporter plasmid and 0.5 μg of pSV40-β-Gal reporter gene was co-transfected with the indicated DNAs. Cells were stimulated with addition of BAPN to a final concentration of 10%, and total cell extracts were prepared after 48 h. The resulting extracts were normalized for β-Gal expression and used in a luciferase activity assay, according to the manufacturer’s instructions. Cells were transfected in 18-mm plastic plates, rinsed once with PBS, washed with 0.1 N NaBH4, and then lysed for 48 h. Localization of the GFP-PDK1 was determined using an Anxiovert 200M fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, N.Y.). Analyses and photographs were performed using Axiosview v.3.1 software; Carl Zeiss MicroImaging, Inc.).
RESULTS

Effect of Suramin on RS485 Cell Phenotype—Treatment of c-Ha-ras-transformed NIH3T3 cells (RS485 cell line) with suramin leads to the induction of lysyl oxidase (29). Here we investigated the effects of suramin on the rate of growth and morphology of RS485 cells, as an initial measure of cell phenotype. Cells were plated in 6-well plates and cultured for 24 h and then grown in the continuous presence of 0, 100, 125, or 150 μM suramin. As control, the growth of phenotypically normal NIH3T3 cells in the absence of suramin was assayed at the same time. Cell growth was determined by daily crystal violet staining of replicate wells (Fig. 1). RS485 cells grew more rapidly than NIH3T3 cells, as expected (30). Suramin significantly decreased the growth rate in a dose-dependent manner. Data show that 100, 125, and 150 μM suramin decreased the growth rate by 38, 49, and 56% respectively, calculated from linear regression analyses of log of absorbance versus time. The growth rate of RS485 cells treated with 150 μM suramin was similar to that of NIH3T3 cells. Furthermore, treatment with suramin caused a dose-dependent change in the morphology of RS485 cells with cells appearing less transformed, i.e. flatter and contact inhibited in the presence of 150 μM suramin (inset, Fig. 1).

We next determined the effects of suramin on cell cycle progression of RS485 cell cultures. As shown in Table I, fluorescence-activated cell sorting (FACS) indicated that treatment with 0 or 150 μM suramin increased the percentage of RS485 cells in G1 and decreased that in S phase in a dose-dependent manner. The proportions of cells in G1 and S phases in cultures treated with 150 μM suramin are nearly identical to untreated phenotypically normal NIH3T3 cells (Table I). Thus, treatment with 150 μM suramin changed the morphology, cell cycle, and growth rate of RS485 cells resulting in a more normal phenotype.

Inhibition of Lysyl Oxidase Expression Reduces Phenotypic Reversion Induced by Suramin—Data presented above indicate that suramin causes phenotypic changes in RS485 cells. To determine that the suramin-induced phenotypic changes depend upon lysyl oxidase expression, we generated stable antisense lysyl oxidase transfected RS485 cell clones. Nine antisense lysyl oxidase transfected clones and six empty vector transfected clones were photographed with a phase contrast microscope (Zeiss) equipped with a conventional Nikon camera.

![Table I](image)

Table I

| Cell type     | G1 % | S % | G2/M % |
|---------------|------|-----|--------|
| RS485         | 43.3 ± 3.6 | 30.8 ± 4.6 | 25.8 ± 1.1 |
| RS485 with 100 μM suramin | 59.4 ± 1.3 | 21.5 ± 2.4 | 19.1 ± 7.9 |
| RS485 with 150 μM suramin | 67.4 ± 0.2 | 19.0 ± 1.7 | 13.6 ± 1.9 |
| NIH3T3        | 66.2 ± 2.1 | 17.4 ± 0.4 | 16.4 ± 3.0 |

![Fig. 1](image) Effects of suramin on growth of RS485 cells. Cells were plated in 6-well plates (35,000 cells/well) and after 24 h the medium was changed to contain either 0 μM (○), 100 μM (▲), 125 μM (●), or 150 μM (□) suramin. Alternatively NIH3T3 cells were grown without suramin (■). At the indicated times, cells were stained with crystal violet, quantitated by spectrophotometry at 590 nm, and growth curves obtained. Each data point is the average of three determinations ± S.D. Inset, morphology of RS485 cells grown in the absence (−Suramin) or constant presence of 150 μM suramin (+Suramin) until confluent. Cultures were photographed with a phase contrast microscope (Zeiss) equipped with a conventional Nikon camera.

![Fig. 2](image) Effect of suramin on the cell cycle progression of antisense lysyl oxidase transfected RS485 cells. Antisense lysyl oxidase transfected and empty vector transfected cells were grown in the presence of 0 or 150 μM suramin for 24 h. Cells (1.5–2 × 10⁶) were fixed in 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. Data shown are the averages ± S.D. of experiments performed three times.

To confirm that lysyl oxidase expression is actually diminished by antisense lysyl oxidase transfection, the ability of 150 μM suramin to induce low steady state mRNA levels of lysyl oxidase in clones was assessed after 24 h of treatment by Northern blot analysis with normalization to 18 S rRNA signals. Suramin treatment led to an average 8-fold increase in lysyl oxidase mRNA levels in the six empty vector-transfected clones assayed, consistent with previous studies on non-transfected RS485 cells (29). In contrast, an average 1.8-fold in-
crease in lysyl oxidase mRNA levels occurred in the nine antisense lysyl oxidase transfected clones. A Northern blot of RNA from representative empty vector and antisense transfected clones is shown in Fig. 3. Thus, the induction of steady state lysyl oxidase mRNA levels by suramin is inhibited by the antisense lysyl oxidase transfection, as expected. Taken together, these data demonstrate that antisense lysyl oxidase transfected cells have significantly diminished suramin-induced cell cycle changes compared with those of empty-vector transfected or non-transfected RS485 cells. These data indicate that lysyl oxidase expression plays a role in mediating the phenotypic effects of suramin on RS485 cells.

**BAPN-mediated Inhibition of Lysyl Oxidase Enzyme Activity Fails to Prevent Phenotypic Reversion Induced by Suramin**—It is generally assumed, although never directly tested, that lysyl oxidase-dependent phenotypic reversion and tumor suppressor activity depends on its enzyme activity. To directly test the role of lysyl oxidase enzyme activity, we measured the effects of the lysyl oxidase inhibitor, BAPN (43), on the phenotypic changes in RS485 cells following treatment with 150 μM suramin. We have previously shown that suramin induces lysyl oxidase activity by about 2.5-fold (29). RS485 cell growth was assessed in the absence or presence of either 150 μM suramin or 400 μM BAPN, or of both 150 μM suramin and 400 μM BAPN. As shown in Fig. 4A, BAPN did not affect the growth rate of PR4 cells. Furthermore, BAPN did not change the morphology of PR4 cells (data not shown). Assays of PR4 cell culture media confirmed that cells grown without BAPN produce easily detectable lysyl oxidase enzyme activity (14,000 ± 400 dpm × 10^6 cells), whereas no lysyl oxidase enzyme activity was detected in the medium of cultures grown at the same time in the continuous presence of both 200 μM and 400 μM BAPN using a highly sensitive assay for lysyl oxidase enzyme activity (4) (data not shown). Taken together, these findings show that growth inhibition of RS485 cells by suramin does not depend on lysyl oxidase enzyme activity. Similarly, inhibition of lysyl oxidase enzyme activity does not affect growth of stable phenotypic revertants that require lysyl oxidase expression for the normal cell phenotype.

**Lysyl Oxidase Propeptide and Not the Active Enzyme Causes Phenotypic Reversion of Antisense Lysyl Oxidase-transfected PR4 Cells (AS-3B) and RS485 Cells**—The biosynthesis of lysyl oxidase includes extracellular proteolysis of 48–50 kDa prolyl oxidase by procollagen C-proteinases to release the 30 kDa lysyl oxidase enzyme and an 18 kDa cationic propeptide. The question, therefore, arises as to whether the released propeptide contributes to phenotypic reversion. To determine the effect of recombinant rat lysyl oxidase propeptide on the phenotype, we first chose to study the effect of the lysyl oxidase propeptide on cell cycle parameters of AS-3B- and RS485-transformed cell lines. AS-3B cells are PR4 cells transformed by stable transfection with antisense lysyl oxidase expression...
subjected to cell cycle analysis. For AS-3B cells (Table II) the effect was observed. Data shown are the averages ± S.D. of experiments performed three times.

| Cell type            | G1  | S    | G2M |
|----------------------|-----|------|-----|
| AS-3B                | 74.4 ± 1.0 | 14.9 ± 1.2 | 10.9 ± 0.8 |
| AS-3B with 1 μg propeptide | 74.7 ± 0.9 | 12.2 ± 0.5 | 13.1 ± 1.2 |
| AS-3B with 5 μg propeptide | 77.6 ± 2.0 | 10.61 ± 1.7 | 1.8 ± 1.6 |
| AS-3B with 10 μg propeptide | 79.9 ± 1.3 | 9.29 ± 1.3 | 10.9 ± 0.5 |

vector (9, 10) and should be sensitive to features of lysyl oxidase that cause phenotypic reversion. Cells were plated on 6-well plates that had been coated with 0, 1, 5, or 10 μg of propeptide per well. This experimental approach was taken due to the poor solubility of the propeptide in cell culture media and physiologic buffers. After 4 days, cells (1–2 × 10⁵) were harvested and subjected to cell cycle analysis. For AS-3B cells (Table II) the percentage of cells in G1 phase increased in the presence of the propeptide by about 6% while the percentage of cells in S phase decreased by 5.7%, suggesting that the lysyl oxidase propeptide has a role in altering the cell cycle of AS-3B cells. Similarly, in RS485 cells (Table III) the percentage of cells in G1 phase increased by 7.6% in the presence of the propeptide, with a corresponding decrease in the percentage of cells in S phase. Results suggest that propeptide affects cell cycle progression of both AS-3B and RS485 cells.

We next investigated the effects of the lysyl oxidase propeptide and of the mature 30 kDa enzyme on the growth of AS-3B and RS485 cells using coated 24-well plates. As shown in Fig. 5, A and B, propeptide decreased the growth of both cell lines in a dose-dependent manner. Linear regression analyses of plots of the log of absorbance versus time demonstrated dose-dependent growth inhibition of 0.9, 3.1, 3.8, and 11.0% for AS-3B cells and 1.0, 5.6, 8.9, and 11.9% for RS485 cells with 0.2, 1, 2, or 4 μg of lysyl oxidase propeptide, respectively. Moreover, the lysyl oxidase propeptide did not affect the plating efficiency of these cells, as initial crystal violet absorbance values were essentially identical for cells plated on propeptide compared with no propeptide (Fig. 5). No obvious effect of propeptide on cell morphology was observed. As an additional control in Fig. 5A, AS-3B cells were grown at the same time on mature 30 kDa lysyl oxidase enzyme. In contrast to the effects of the lysyl oxidase propeptide, lysyl oxidase enzyme did not inhibit the growth rate of AS-3B cells (Fig. 5A). In fact, growth on lysyl oxidase enzyme appeared to be slightly increased compared with the control. As seen in Fig. 5A, this is because of higher plating efficiency of cells on the mature enzyme. Linear regression analyses showed that the rate of cell growth on lysyl oxidase enzyme was essentially unaffected (increased by 2%), even though plating efficiency was increased. Taken together, the data indicate that the lysyl oxidase propeptide has a specific inhibitory effect on cell growth and cell cycle progression that contributes to phenotypic reversion.

Lysoy Oxidase Propeptide, and Not the Active Enzyme, Inhibits Growth of RS485 Cells in Soft Agar—A hallmark of transformed cells is the ability to grow in soft agar and to form colonies, whereas non-transformed cells are unable to grow when suspended in soft agar. The respective effects of active 30 kDa lysyl oxidase enzyme, and of the 18 kDa lysyl oxidase propeptide versus vehicle control on the ability of RS485 cells to grow in soft agar were determined. Lysyl oxidase propeptide was strongly inhibitory, whereas the 30 kDa lysyl oxidase enzyme was unable to inhibit growth of RS485 cells in soft agar (Fig. 6). In two separate experiments an average 80% reduction in colony formation was observed. Lysyl oxidase-mediated reversion appears to be selective for ras-mediated transformation. To investigate the specificity of the propeptide, its growth inhibitory on c-myc-transformed M158 fibroblasts was determined. Neither lysyl oxidase propeptide nor the 30 kDa lysyl oxidase enzyme inhibited the growth in soft agar of c-myc-transformed cells (9, 10).

Lysyl Oxidase Propeptide and Akt-mediated NF-κB Activity—NF-κB is highly activated in ras-transformed NIH3T3 cells, and transfection with a full-length lysyl oxidase expres-
LO pro-peptide or vehicle control. Values per field were averaged as percent of vehicle control.

The LO propeptide and the enzyme were counted in three independent fields, and values per field expressed as percent of vehicle control.

Inhibited the lysyl oxidase propeptide, and not the lysyl oxidase enzyme, inhibits the PI3K/Akt pathway to revert the phenotype of ras-transformed fibroblasts. This report shows for the first time that the ability of lysyl oxidase to revert the phenotype of ras-transformed fibroblasts depends substantially on the propeptide domain, and not on lysyl oxidase enzyme activity. Since diminished lysyl oxidase expression in some way contributes to the transformed phenotype, it has generally been assumed that lysyl oxidase enzyme activity is related to the tumor suppressor activity of lysyl oxidase, and, therefore, that diminished lysyl oxidase activity promotes the transformed phenotype. However, BAPN, the specific inhibitor of lysyl oxidase enzyme activity, did not prevent suramin-mediated reversion of the transformed phenotype, which is accompanied by increased lysyl oxidase expression.

**DISCUSSION**

This report shows for the first time that the ability of lysyl oxidase to revert the phenotype of ras-transformed fibroblasts depends substantially on the propeptide domain, and not on lysyl oxidase enzyme activity. Since diminished lysyl oxidase expression in some way contributes to the transformed phenotype, it has generally been assumed that lysyl oxidase enzyme activity is related to the tumor suppressor activity of lysyl oxidase, and, therefore, that diminished lysyl oxidase activity promotes the transformed phenotype. However, BAPN, the specific inhibitor of lysyl oxidase enzyme activity, did not prevent suramin-mediated reversion of the transformed phenotype, which is accompanied by increased lysyl oxidase expression.
RS485 cells, and in RS485 cells transfected with the expression vector plasma membrane localization of GFP-PDK1 clearly present only in as described under vectors (upper panel of GFP-PDK1 construct (induced by Ras. NIH3T3 and RS485 cells were transfected with 2 μg of GFP-PDK1 construct (upper panel). RS485 cells were co-transfected with either lysyl oxidase propeptide or lysyl oxidase enzyme expression vectors (lower panel). Cells were incubated 48 h in 10% FBS and PDK1 cellular localization was photographed using fluorescence microscopy as described under “Experimental Procedures.” The arrows mark the plasma membrane localization of GFP-PDK1 clearly present only in RS485 cells, and in RS485 cells transfected with the expression vector for mature lysyl oxidase enzyme.

These findings were confirmed in the stable phenotypic revertant cell line PR4, that requires lysyl oxidase expression for normal phenotype maintenance; yet inhibition of lysyl oxidase activity with BAPN failed to re-transform these cells. Similarly, BAPN failed to block the ability of ectopic lysyl oxidase expression to prevent growth of ras-transformed fibroblasts in soft agar (data not shown). The lack of effect of BAPN on lysyl oxidase-dependent phenotype control is interesting. Intracellular localization of mature lysyl oxidase has been shown to occur via normal extracellular processing of pro-lysyl oxidase, followed by uptake of mature lysyl oxidase (46). Given that BAPN is an irreversible inhibitor of lysyl oxidase (43), it follows that both extracellular and intracellular lysyl oxidase activity are susceptible to inhibition by BAPN. These findings, therefore suggest that neither extracellular nor intracellular lysyl oxidase activity contribute significantly to inhibiting the transformed cell phenotype.

The importance of lysyl oxidase expression in maintaining a normal cell phenotype in suramin treated RS485 cells was supported by antisense transfection studies. Most important, using recombinant lysyl oxidase propeptide, we demonstrated that the lysyl oxidase propeptide itself directly stimulates phenotypic reversion of ras-transformed cells, as judged by rate of proliferation, cell cycle colony formation in soft agar, and PDK1/Akt signaling to activate NF-kB. This contrasts with the absence of an effect of the lysyl oxidase enzyme on both the growth rate of AS-3B cells, and on colony formation in soft agar of RS485 cells. These studies identify an activity of the lysyl oxidase propeptide that may ultimately prove to be of therapeutic significance in the treatment of cancers in which ras-dependent pathways are abnormally active.

A concept that has gained increasing experimental support is that many proteins have multiple biological functions (47). A recently reported example is the housekeeping cytoplasmic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has been unexpectedly found in the nucleus as a component of a transcription complex (48). It is unknown whether GAPDH enzyme activity plays a role in its activity as a transcription complex component, though its co-activator activity does depend on NAD+ binding. Lens crystallins are structural proteins found in the lens that in other contexts serve as enzymes (49). Similarly, there is an increasing appreciation for biological activities of propeptides of structural proteins that are released as a result of biosynthetic proteolytic processing and maturation. C-propeptides of type I and type II collagen are ligands for α5β1 integrins and they inhibit collagen gene transcription (50–53). The C-propeptide of type I collagen promotes attachment of osteoblasts, and is chemotactic for endothelial cells (54, 55). A variant of the N-terminal propeptides of type II collagen binds and modulates the activity of TGF-β1 and BMP-2 in developing cartilage (56). Endostatin is a 20 kDa cationic protein derived from C-terminal extensions of type XVIII procollagen that inhibits angiogenesis (57), an activity that is receiving much attention as a therapeutic approach to treat cancer. The finding reported here that the lysyl oxidase propeptide has biological function as an inhibitor of cell transformation provides a new and important example of a distinct biological activity derived from an extracellular protein precursor.

Structural features of the lysyl oxidase propeptide are interesting. The biosynthesis of lysyl oxidase occurs by secretion of a 50 kDa precursor, followed by extracellular proteolytic processing to form active 30 kDa lysyl oxidase and the 18–20 kDa propeptide (5–7). Unlike the anionic C-terminal region of pro-lysyl oxidase that becomes the active enzyme after processing (6, 7), the N-terminal propeptide region is rich in arginine and is cationic with a calculated pI of 12.5 for the mouse, rat, and human proteins. We hypothesize that the highly basic character of the lysyl oxidase propeptide could facilitate its uptake by cells where it might exert its biological function, possibly entering cells even in the absence of a specific receptor. Cell membranes are permeable to arginine-rich basic proteins, and uptake of these basic proteins is mediated by heparin sulfate proteoglycans (58). The arginine-rich highly basic propeptide region of lysyl oxidase is less well conserved between species than the mature enzyme (59), but it contains blocks of 33 and 37 amino acids residues in length, respectively, that are nearly perfectly conserved between mouse and human, and highly conserved in chicken lysyl oxidase. These regions are residues 26–59 and 77–114, respectively, in the mouse lysyl oxidase sequence. This high degree of similarity suggests that biological activities of the lysyl oxidase propeptide reside in these conserved sequences. Lysyl oxidase is a member of a multigene family, and it is notable that the sequence of the lysyl oxidase propeptide region is not well conserved among other lysyl oxidase family members, whereas the catalytic domains are well conserved. Lysyl oxidase itself, and not the lysyl oxidase like genes, has been consistently identified in screens for tumor suppressors and is expressed at low levels in transformed cells and at higher levels in phenotypically normal cells (9, 12). The finding of phenotype modulating activities occurring in regions of lysyl oxidase that are located in the unique propeptide domain may help to explain why lysyl oxidase itself is a tumor suppressor. Comparisons of the lysyl oxidase propeptide sequence with data bases have so far not revealed clues regarding the mechanisms by which the lysyl oxidase propeptide functions to inhibit cell transformation. However, our data do suggest that the lysyl oxidase propeptide inhibits the ras-dependent PI3K/PDK1/Akt signal transduction pathway similar to what was found in ras-transformed cells transfected with full-length lysyl oxidase (18). It is interesting to note that NF-κB activity in propeptide transfected cells was inhibited as early as 48 h after transfection, whereas this effect was not observed until 72 h after transfection with the full-length lysyl oxidase proenzyme. Although our data so far do not directly address whether full length lysyl oxidase proenzyme that contains the propeptide
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is active in promoting phenotypic reversion, these results suggest that it is the released propeptide itself that inhibits NF-

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