The Lysyl Oxidase Pro-peptide Attenuates Fibronectin-mediated Activation of Focal Adhesion Kinase and p130Cas in Breast Cancer Cells*

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Yingshe Zhao†, Chengyin Min‡, Siddharth R. Vora§, Philip C. Trackman§, Gail E. Sonenshein§, and Kathrin H. Kirsch††

From the †Department of Biochemistry, Boston University School of Medicine and the ‡Division of Oral Biology, Boston University Goldman School of Dental Medicine, Boston, Massachusetts 02118

The lysyl oxidase (LOX) gene encodes an enzyme (LOX) critical for extracellular matrix maturation. The LOX gene has also been shown to inhibit the transforming activity of Ras oncogene signaling. In particular, the pro-peptide domain (LOX-PP) released from the secreted precursor protein (Pro-LOX) was found to inhibit the transformed phenotype of breast, lung, and pancreatic cancer cells. However, the mechanisms of action of LOX-PP remained to be determined. Here, the ability of LOX-PP to attenuate the integrin signaling pathway, which leads to phosphorylation of focal adhesion kinase (FAK), and the activation of its downstream target p130Cas, was determined. In NF639 breast cancer cells driven by Her-2/neu, which signals via Ras, ectopic Pro-LOX and LOX-PP expression inhibited fibronectin-stimulated protein tyrosine phosphorylation. Importantly, phosphorylation of FAK on Tyr-397 and Tyr-576, via Ras, ectopic Pro-LOX and LOX-PP expression inhibited fibronectin-activated haptotaxis were decreased. Interestingly, expression of mature LOX enzyme enhanced fibronectin-stimulated integrin signaling. Of note, treatment with recombinant LOX-PP selectively reduced fibronectin-mediated haptotaxis of NF639, MDA-MB-231, and Hs578T breast cancer cells. Thus, evidence is provided that one mechanism of action of LOX-PP tumor suppression is to block fibronectin-stimulated signaling and cell migration.

The lysyl oxidase (LOX) gene family is comprised of five members LOX, LOXL1, LOXL2, LOXL3, and LOXL4, which encode enzymes that modify extracellular matrix (ECM) proteins to promote their cross-linking and deposition (1). The LOX gene is the best characterized and codes for the synthesis of a secreted 50-kDa glycosylated pro-enzyme (Pro-LOX). Pro-LOX is extracellularly processed by proteolytic cleavage to a mature active 32-kDa enzyme (LOX) and an 18-kDa pro-peptide (LOX-PP) by the procollagen C proteinases bone morphogenetic protein-1 (BMP-1), and the related tolloid-like proteins TLL1 and TLL2 (2–4). In murine Pro-LOX, proteolytic processing occurs between amino acids Gly-162 and Asp-163, generating LOX-PP containing 141 amino acids (5). LOX-PP contains two consensus N-glycosylation sites, Asn-91 and Asn-138 (murine sequence) (2) and several O-glycosylation sites.3 LOX-PP does not contain any known protein domains, and structural prediction analysis indicates that LOX-PP assembles as an intrinsically disordered protein (6). Among the LOX family members, the C-terminal ends encode the enzyme domain and are highly conserved, whereas the N-terminal ends that encode the pro-peptide region have variable sequences. Based on structural and sequence similarities of the pro-peptide regions, the LOX family members can be divided into two subgroups: LOXL2, LOXL3, and LOXL4 as one group whose pro-peptide regions contain four scavenger receptor cysteine-rich domains, and LOX and LOXL1 as a separate group with much simpler and smaller pro-peptide region containing no cysteine residues (reviewed in Ref. 1). In contrast to Pro-LOX, the exact maturation site of Pro-LOXL1 is still unidentified.

LOX is essential in the formation of blood vessels and in maintaining their normal characteristics (7–9). Up-regulation of LOX expression has been described in stromal cells that surround ductal breast and broncho-pulmonary carcinomas (10). Expression of the LOX gene was found to inhibit the transforming activity of the Ras oncogene in NIH 3T3 fibroblasts and hence was named the “ras reversion” gene (rrg) (11, 12). The LOX gene was shown to inhibit growth in soft agar of NIH 3T3 fibroblasts and to attenuate Ras-mediated activation of phosphatidylinositol 3-kinase (PI3K), Akt, and Erk1/2 kinases and NF-κB activation (13). More recently, the rrg activity was mapped to the 18-kDa LOX-PP. Specifically, LOX-PP was shown to inhibit Ras-mediated transformation of fibroblasts as determined by reduced growth in soft agar, localization of PDK1 to the membrane, and activation of NF-κB (14). Furthermore, the inhibitory effects of LOX-PP on Ras signaling were extended to breast, pancreatic, and lung cancer cells (6, 14, 15).
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LOX-PP expression in these carcinoma cells reverted Her-2/neu- and Ras-mediated epithelial to mesenchymal transition (EMT), leading to increased expression of E-cadherin and γ-catenin, and reduced levels of Snail, vimentin, and/or BCL-2 (7, 15). Furthermore, LOX-PP expression reduced tumor formation in a xenograft model by Her-2/neu-overexpressing NF639 cells (6).

Acquisition of the ability to invade the ECM is essential to EMT. The ECM has multiple mechanical and signaling functions. The ECM defines interfaces between tissues, provides a scaffold for cell traction, and a substrate for cell migration and adhesion. It is composed of a complex of proteins such as collagen, fibronectin, and laminin, which can interact and bind various growth factors (16). Fibronectin is of particular interest because it was recently shown to interact with the C terminus of Pro-LOX (17). Binding of fibronectin to its receptors (e.g. integrins α5β1 or αvβ3) stimulates the tyrosine phosphorylation of cellular proteins, in particular that of focal adhesion kinase (FAK) (18). Little is known about the mechanism of action of LOX-PP. Here, we have asked whether the tumor suppressor activity of LOX-PP attenuates the activation of the integrin signaling pathway in breast cancer cells. We report that LOX-PP attenuates FAK signaling and activation of its downstream target p130Cas and is a robust inhibitor of fibronectin-stimulated cell migration.

EXPERIMENTAL PROCEDURES

Cell Lines—Preparation and culture conditions of stable infectants of NF639 cells with control empty vector (EV) pC4bsR(TO), Pro-LOX, LOX, and LOX-PP have been described previously (6). Human MDA-MB-231 breast carcinoma cells, which contain a mutated, constitutively active K-Ras (19) and human Hs578T breast carcinoma cells, which contain a mutated, constitutively active H-Ras (20), were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mm glutamine, and penicillin/streptomycin, as described previously (21).

Antibodies and Recombinant Protein—Mouse monoclonal antibodies (mAbs) directed against FAK-pY397 and FAK-pY579 were purchased from BioSource. Antibodies against p130Cas, pY249 and phospho-ERK1/2Thr202/Thr204 were purified from Cell Signaling. FAK-pY397, PY-99, and p130Cas-pY249 and phospho-ERK1/2Thr202/Thr204 were purchased from BioSource. Antibodies against FAK-pY397 and FAK-pY579 were purchased from BioSource. Antibodies against FAK-pY397 and FAK-pY579 were purchased from BioSource. Antibodies against FAK-pY397 and FAK-pY579 were purchased from BioSource. Antibodies against FAK-pY397 and FAK-pY579 were purchased from BioSource.

Haptotaxis Cell Migration Assay—Cell migration experiments were performed using 8-μm pore Transwell filters (Costar), which were coated on the underside with 50 μg/ml human fibronectin, 50 μg/ml laminin or 5 μg/ml vitronectin (BD Biosciences), for 1 h at room temperature. Membranes were washed with 1× phosphate-buffered saline (PBS), air-dried, and stored at 4 °C until use. Cells (1 × 10^6) were suspended, in triplicate, in DMEM supplemented with 0.5% bovine serum albumin (BSA) and layered in the upper chamber. For blocking protein synthesis, cells were pretreated with 50 μg/ml cycloheximide (CHX) (Sigma-Aldrich) for 30 min. The cells were washed twice with PBS, trypsinized, and processed as above. The lower compartment contained 500 μl of DMEM supplemented with 0.5% BSA. After the indicated incubation period, the upper surface of the filter was wiped with a cotton-tipped applicator to remove non-migratory cell. Cells that had migrated through the filter pores and attached to the underside of the filter were fixed and stained in methanol containing 0.5% crystal violet. The dye was extracted by incubation in 1× PBS, 2% SDS for 10 min. Migration was quantified by determining the A_{470} nm. Results obtained with EV cells were set to 100% and used as reference. Migration of Pro-LOX, LOX, or LOX-PP cells was expressed as a percentage of the control. To determine the effects of addition of rLOX-PP on migration, cells were pretreated for 24 h with purified rLOX-PP at final concentrations of 0, 0.9, and 4.5 μg/ml corresponding to ~0, 50, or 250 nm, respectively. Subsequently, 1 × 10^6 cells were suspended in DMEM supplemented with 0.5% BSA and purified rLOX-PP at a final concentration of 0 (control), 50, 250 nm, and layered, in triplicate, into the upper chamber and incubated at 37 °C for 3–5 h, and processed as above. Control cells were set to 100%. Data were generated from a minimum of three independent experiments. For statistical analyses, a Student’s t test was performed. p values <0.05 were considered statistically significant.

Integrin Stimulation—Three percent methylcellulose–DMEM medium supplemented with 10% FBS was prepared as described (23). Briefly, autoclaved methylcellulose (4000 cP viscosity) (Sigma-Aldrich) was dispersed in hot deionized H2O, supplemented with 0.4% NaHCO3, and cooled to room temperature. This solution was mixed with equal volume of 2× DMEM and solubilized overnight at 4 °C. The methylcellulose–DMEM mix was supplemented with 10% FBS and 2 μg/ml doxycycline (Sigma-Aldrich). NF639 stable infectants were treated with 2 μg/ml doxycycline for 48 h to induce transgene expression. Cells were trypsinized and washed in complete medium. Approximately 1.5 × 10^6 cells per 25 ml of methylcellulose–DMEM were incubated in the presence of doxycycline at 37 °C for 24 h. Subsequently, suspended cells were recovered and seeded at a density of 1.5 × 10^6 cells per 100-mm dish coated with fibronectin, laminin, or vitronectin (BD Biosciences) for 1.5 h. For CHX treatment studies, cells recovered from methylcellulose–DMEM suspension culture were incubated in serum-free DMEM with or without 50 μg/ml CHX for 30 min. Following incubation, cells were washed twice and seeded in fibronectin-coated 100-mm dishes for 1.5 h, as above. Adherent cells were washed twice with PBS and cellular proteins were extracted by lysing with RIPA buffer (50 mM Tris- HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitors (1 mM sodium vanadate, 10 mM sodium fluoride).

Immunoprecipitation and Western Blotting—Cultured cells were washed twice with cold 1× PBS, and lysed in ice-cold RIPA buffer (as above) for 30 min on ice followed by centrifu-
gation at 16,000 \( \times \) g at 4 °C for 30 min yielding whole cell extracts (WCE). Expression of the V5-tagged LOX-PP in cell culture medium (MED) was detected by immunoprecipitation as described previously (6). Immunoprecipitates or WCE \( (20 \mu g) \) were subjected to immunoblotting, as described (24).

Preparation of Triton X-100-soluble and -insoluble Protein Extracts—Cells, washed twice with 1× PBS, were incubated with 1 ml of CSK buffer \( (0.5\% \text{Triton X-100, 10 mM Tris-HCl, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl}_2) \) supplemented with phosphatase and protease inhibitors at 4 °C for 15 min. The lysed cells were collected and centrifuged at 13,600 \( \times \) g at 4 °C for 30 min. The supernatant was defined as the Triton X-100 soluble fraction. The cell pellet was washed once with 200 \( \mu l \) of CSK buffer and extracted with 1 ml of SDS buffer \( (1\% \text{ SDS, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA}) \) and boiled for 10 min. The cell debris was removed by centrifugation at 13,600 \( \times \) g at 4 °C for 30 min. The supernatant was defined as the Triton X-100-insoluble fraction. For each sample, a 10-\( \mu g \) protein aliquot of soluble fraction and an equal volume (in reference to the concentration of the soluble fraction) of insoluble fraction were separated by SDS-PAGE on an 8% resolving gel.

Phase Contrast Microscopy—Culture plates \( (24\text{-well}) \) (Greiner Labortecnik) were coated with ECM proteins at the following concentrations: 50 \( \mu g/ml \) fibronectin, 50 \( \mu g/ml \) laminin, or 5 \( \mu g/ml \) vitronectin at room temperature for 1 h. Subsequently, the coated wells were washed twice with 1× PBS and used immediately or stored at 4 °C. NF639 or MDA-MB-231 cells were trypsinized, and washed in complete medium. Cells were resuspended in complete medium and seeded at 1 \( \times \) 10\(^5\)/well for 1.5 h and 3 h. Cells were inspected by using an inverted Axiovert 200 m microscope (Carl Zeiss), and images taken with a CCD ORCA-ER camera (Hamamatsu) attached to the inverted microscope.

Immunofluorescence—NF639 or MDA-MB-231 cells \( (1 \times 10^5) \) were plated in 12-well plates with or without rLOX-PP \( (250 \text{ nm}) \) for 24 h. Subsequently, the cells were trypsinized, washed once with complete DMEM and once with serum-free DMEM. Cells \( (1 \times 10^4) \) were then plated on fibronectin-coated Lab-Tek II chamber slides (Nalge Nunc International) and cultured in DMEM supplemented with 2.5% FBS and with or without rLOX-PP \( (250 \text{ nm}) \) for 24 h. Cells were fixed with 3% formaldehyde/PBS at room temperature for 30 min and permeabilized with 0.2% Triton X-100/PBS at room temperature for 2–5 min. Nonspecific binding sites were blocked with 50 mM glycine/PBS for 10 min. Cells were stained with PY99 antibodies \( (1:200) \) in 0.2% gelatin/PBS or rhodamine-labeled phalloidin \( (1:1000) \) at room temperature for 1 h. After four washes, cells were incubated with secondary anti-mouse Alexa Fluor 488 antibodies \( (1:1000) \). Samples were inspected by using an Axiovert 200 M microscope (Carl Zeiss) and images taken with an ORCA-ER camera (Hamamatsu).

**RESULTS**

**LOX-PP Attenuates Fibronectin-stimulated Tyrosine Phosphorylation of Cellular Proteins**—To elucidate the role of LOX-PP in adhesion-mediated cell signaling, we utilized the recently established Her-2/neu-transformed NF639 breast cancer cell populations expressing Pro-LOX, LOX, or LOX-PP or containing control EV DNA driven by a doxycycline-inducible promoter (6). Induction of recombinant peptide expression following doxycycline treatment was confirmed by Western blot analysis of WCE to detect proteins in the intracellular compartment, and of culture media to detect secreted proteins (Fig. 1A). Expression of LOX is restricted to the intracellular compartment, while Pro-LOX and LOX-PP are secreted as has been described previously (6, 25).

Engagement of fibronectin with its integrin receptors results in rapid and robust activation of non-receptor tyrosine kinases. We first tested whether LOX-PP reduces integrin-mediated induction of tyrosine kinase activity. Cells were treated with doxycycline for 48 h, and subsequently incubated in semi-solid methylcellulose containing medium \( (\text{suspension culture}) \) for an additional 24 h, to de-activate integrin signaling (26). Cells were recovered from the methylcellulose containing medium and lysed immediately \( \text{(SUSP)} \) or plated on fibronectin-coated dishes for 1.5 h before cell lysis (FN), as described under “Experimental Procedures.” Western blot analysis with phosphotyrosine-specific antibodies consistently revealed an increase in appearance of a major phosphorylated protein species of \( \sim 120\)-kDa molecular mass \( (\text{indicated with an arrowhead}) \) in all lysates of cells plated on fibronectin compared with those in suspension \( (\text{Fig. 1B, upper panel}). \) This phosphorylated band may represent several proteins involved in integrin signaling including FAK and p130\(^{crk}\). Attenuated phosphorylation of this band was seen in NF639 Pro-LOX- and LOX-PP-expressing cells compared with NF639 EV cells. Analysis of a total of three independent experiments revealed Pro-LOX and LOX-PP resulted in an approximate 40% reduction \( (p < 0.0001 \text{ and } p < 0.001, \text{respectively}) \) in tyrosine phosphorylation when normalized to the β-actin loading control \( (\text{Fig. 1B, lower panel}). \) Consistent with the hypothesis that the pro-peptide region attenuates integrin signaling, in contrast, tyrosine phosphorylation in NF639 LOX cells was comparable to NF639 EV cells. Notably, inhibition of protein synthesis following addition of 50 \( \mu g/ml \) CHX for 30 min failed to prevent the ability of LOX-PP to inhibit adhesion-mediated induction of phosphotyrosine, suggesting newly synthesized proteins are not required \( (\text{Fig. 1C}). \)

As described above, the ECM is composed of a complex of proteins. Thus we compared fibronectin-stimulated tyrosine phosphorylation of NF639 cells with that of stimulation by laminin and vitronectin. Western blot analysis of WCE with phosphotyrosine-specific antibodies revealed an increase in appearance of the major phosphorylated protein species of \( \sim 120\)-kDa molecular mass when cells were plated on fibronectin for 1.5 h. In contrast, no or only a weak increase in tyrosine phosphorylation was observed for laminin or vitronectin stimulation, respectively, compared with those in suspension \( (\text{Fig. 1D, upper panel}). \) These differences correlate with the ability of NF639 cells to spread/adhere on these three adhesive proteins. While cell adhesion/spreading over a period of 1.5 h was superior on fibronectin-coated surfaces, it was less pronounced on vitronectin and completely absent on laminin \( (\text{Fig. 1D, lower panel}). \) These data suggest that fibronectin plays a major role in integrin activation of NF639 cells.
by Pro-LOX and LOX-PP is due to reduced activation of FAK. Cells were again induced with doxycycline, suspended in methylcellulose, and then plated on fibronectin as above. Western blot analysis of WCE was performed using antibodies that specifically recognize these phosphorylated tyrosines on FAK. Phosphorylation of FAK on its auto-phosphorylation site Tyr-397 upon adhesion to fibronectin was significantly reduced by Pro-LOX that contains the LOX-PP domain, and by LOX-PP itself (Fig. 2A, upper panel). When the data from this and two duplicate experiments were scanned and normalized to the β-actin loading control, a ~50% reduction of phosphorylation was seen in cells expressing Pro-LOX and LOX-PP (p < 0.0001 and p < 0.001, respectively) (Fig. 2A, bottom panel). Similarly, integrin engagement to fibronectin induced phosphorylation of the activation tyrosine Tyr-576, and LOX-PP substantially reduced this phosphorylation by ~25% (Fig. 2B, upper panel). In contrast, LOX expression failed to reduce phosphorylation on either site Tyr-397 or Tyr-576. Furthermore, no change in the phosphorylation status of the Tyr-925 Grb2 binding site was detected upon cell attachment to fibronectin-coated plates, i.e. the same degree of phosphorylation was noted in all conditions, and was not altered by stimulation with fibronectin or expression of LOX-PP (Fig. 2B, bottom panel). Thus, these data suggest that LOX-PP interferes with stimulation of FAK signaling by fibronectin, in particular selectively attenuating phosphorylation of two tyrosine residues: Tyr-397 and Tyr-576.

**LOX-PP Attenuates Fibronectin-stimulated Activation of FAK—**As noted, the phosphorylated band affected by LOX-PP and Pro-LOX may represent one of several proteins involved in integrin signaling, including FAK or p130Cas. FAK, can be phosphorylated at several functional tyrosine sites: Tyr-397, auto-phosphorylation and c-Src-binding site; Tyr-576, activation tyrosine; Tyr-925, Grb2 binding site. Thus, we addressed whether the decrease in tyrosine phosphorylation mediatedLOX-PP Inhibits Integrin Signaling

**FIGURE 1. Pro-LOX and LOX-PP expression inhibits fibronectin-stimulated tyrosine phosphorylation.** A, expression of V5-tagged Pro-LOX, LOX, LOX-PP, and EV control in stable infectants of NF639 cells in the absence (−) or presence (+) of 2 μg/ml doxycycline (DOX) (left panel). Whole cell extracts (WCE, 20 μg of protein) were subjected to Western blot analysis using V5 antibodies (left panel). The culture media (MED, 1 ml) of LOX-PP cells or control NF639-EV cells were collected and subjected to immunoprecipitation as described under “Experimental Procedures.” Western blot analysis was performed with V5 antibodies (right panel). Positions of molecular weight markers are given on the left. B, doxycycline-treated NF639 stable infectants (1.5 × 10⁹ cells per 25 ml of methylcellulose medium) were cultured in suspension for 24 h. Subsequently, suspended cells were recovered and seeded at a density of 1.5 × 10³ cells per 100-mm fibronectin-coated dish for 1.5 h (FN) or lysed immediately (SUSP). Adherent cells were washed twice with 1 × PBS and cellular proteins were extracted by lysing with RIPA buffer. WCE (20 μg of protein) were subjected to Western blotting using antibodies against total phosphorylated tyrosines (P-Tyr) and β-actin (upper panel). Values normalized to β-actin levels are presented as the mean ± S.D. from three independent experiments. EV control was set as 100%. p values were calculated using Student’s t test (lower panel). C, CHX treatment does not alter the activity of LOX-PP. Doxycycline-treated NF639 cells expressing the control EV or LOX-PP were cultured as above. Before seeding onto fibronectin-coated dishes for 1.5 h, cells were incubated with 50 μg/ml CHX for 30 min. WCE (20 μg of protein) were subjected to Western blotting using antibodies against total phosphorylated tyrosines (P-Tyr) and β-actin (upper panel). Values normalized to β-actin levels are presented as the mean ± S.D. from three independent experiments. EV control was set as 100%. p values were calculated using Student’s t test (lower panel). D, integrin activation and cell adhesion/spreading of NF639 cells depends on the ECM proteins. NF639 cells were cultured in suspension for 24 h, as described. Subsequently, recovered cells were seeded at a density of 1.5 × 10³ cells per 100-mm fibronectin- (FN), laminin- (LN), or vitronectin- (VN) coated dish for 1.5 h or lysed immediately (SUSP). WCE (20 μg of protein) were subjected to Western blotting using antibodies against total phosphorylated tyrosines (P-Tyr) and β-actin (upper panel). NF639 were seeded at 1 × 10⁴ well in 24-well plates coated with fibronectin, laminin, or vitronectin as described, and phase contrast images were taken 1.5 h after seeding. Scale bars, 50 μm (lower panel).

**LOX-PP Attenuates Fibronectin-stimulated p130Cas Activation—**Recruitment and phosphorylation of the tyrosine phosphorylated adapter-type protein p130Cas is a critical step in FAK-mediated migration and invasion. To determine whether LOX-PP expression affects the activation status of p130Cas, extracts of fibronectin-stimulated NF639 control EV cells or cells expressing Pro-LOX, LOX, or LOX-PP were subjected to Western blot analysis for their effects on levels of phosphorylated, activated p130Cas.
protein (Fig. 3A, upper panel). Tyrosine phosphorylation of p130Cas was significantly reduced in fibronectin-stimulated Pro-LOX- and LOX-PP-expressing NF639 cells compared with the control cells, whereas LOX failed to reduce phospho-p130Cas levels (Fig. 3A, lower panel).

Phosphorylated p130Cas is localized to the cytoskeleton fraction, in particular to focal adhesions. Therefore, to test whether LOX-PP reduces the translocation of p130Cas to focal adhesions, levels of p130Cas in the Triton X-100 insoluble (I) (cytoskeleton/focal adhesions) versus soluble (S) cell fractions were determined. Most of the p130Cas was present in the Triton X-100-soluble (S) and insoluble (I) fractions were prepared from the indicated NF639 cell population. For each sample, a 10-μg protein aliquot of the soluble fraction and an equal volume of insoluble fraction (in reference to the concentration of the soluble fraction) were analyzed by Western blotting with p130Cas antibodies. Densitometric scanning was performed and the ratio of p130Cas protein in the insoluble versus soluble fractions (I/S, values given below) calculated. This experiment was performed twice with similar results.

FIGURE 3. Pro-LOX and LOX-PP expression inhibit fibronectin-stimulated activation of p130Cas in NF639 cells. A, upper panel, WCE were prepared as described in Fig. 2, and samples (20 μg of protein) were subjected to Western blotting using phosphospecific (p130Cas-pY249), total p130Cas, and β-actin antibodies. Lower panel, values are means ± S.D. from three independent experiments normalized to β-actin. p values were calculated using Student’s t test. B, protein extracts of the Triton X-100 soluble (S) and insoluble (I) fractions were prepared from the indicated NF639 cell population. For each sample, a 10-μg protein aliquot of the soluble fraction and an equal volume of insoluble fraction (in reference to the concentration of the soluble fraction) were analyzed by Western blotting with p130Cas antibodies. Densitometric scanning was performed and the ratio of p130Cas protein in the insoluble versus soluble fractions (I/S, values given below) calculated. This experiment was performed twice with similar results.
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**FIGURE 4.** LOX-PP inhibits fibronectin-stimulated haptotaxis of NF639 cells. A, Boyden chamber filters were coated on the underside with 5 μg/ml fibronectin. After a 48-h period of induction with doxycycline, the indicated NF639 cell populations were subjected to a haptotaxis migration assay for 2.5 h. Random motility was assessed on BSA-coated filters. B, NF639 cell populations were subjected to a haptotaxis migration assay for fibronectin. After a 48-h period of induction with doxycycline, the indicated NF639 cells were subjected to haptotaxis assay in the absence or presence of rLOX-PP. As shown in Fig. 4A, rLOX-PP treatment resulted in a dose-dependent reduction in migration of NF639 cells (27% at the higher dose), consistent with the data obtained with cells overexpressing LOX-PP (Fig. 4B). Furthermore we examined the effect of rLOX-PP on adhesion structures. NF639 cells were pretreated with rLOX-PP (0 and 250 nM) for 24 h, and then plated on fibronectin-coated chamber slides in the presence of 0 and 250 nM rLOX-PP. After an additional 24 h, cells were stained for filamentous actin (F-actin) and tyrosine phosphorylation. A change in morphology was apparent upon treatment with rLOX-PP. NF639-treated cells displayed a more triangular shape (Fig. 5, upper panels). Moreover, analysis of the high magnification images revealed a profoundly reduced concentration of phosphorylated proteins in focal adhesions (Fig. 5, middle and lower panels), supporting our findings that LOX-PP inhibits fibronectin-stimulated tyrosine phosphorylation of FAK and p130Cas (Figs. 2 and 3).

We next examined whether rLOX-PP protein can inhibit migration of the highly invasive human MDA-MB-231 and Hs578T breast cancer cells, which carry a mutated K-RAS and H-RAS gene, respectively (19, 20). Treatment with rLOX-PP resulted in pronounced reduction in migration of these two lines (Fig. 6, A and B). A dose-dependent inhibition of fibronectin-stimulated haptotaxis was seen with MDA-MB-231 and Hs578T cells with a reduction of 51, and 47%, respectively, at the higher dose. Again, no differences in migration were observed when cells were plated on BSA-coated filters. Similar to the NF639 cells, attenuated accumulation of phosphorylated proteins to focal adhesions was seen in rLOX-PP-treated MDA-MB-231 cells (supplemental Fig. S1).

Moreover, attenuation of the haptotaxis by rLOX-PP was specific for fibronectin. When laminin or vitronectin were used as attractant, no significant reduction in the haptotactic response was observed for LOX-PP-treated murine NF639 as well as human MDA-MB-231 breast cancer cells (supplemental Fig. S2A). Furthermore, adhesion/spreading of MDA-MB-231 cells on laminin- or vitronectin-coated surfaces was absent or low, respectively, similar to NF639 cells, which correlates well with a slower migratory response compared with fibronectin stimulation (supplemental Fig. S2, B and C). Thus, these data further suggest that LOX-PP interferes specifically with fibronectin-stimulated cell migration in mouse and human breast cancer cell lines.

**DISCUSSION**

Here we show for the first time that expression of LOX-PP attenuates fibronectin-mediated tyrosine kinase signaling, as determined by reduction in tyrosine-phosphorylated FAK and contributes to diminished migratory response. Interestingly, protein synthesis is not required for inhibitory activity of LOX-PP as pretreatment of cells with CHX did not alter the effect of LOX-PP on haptotaxis (Fig. 4B), suggesting that LOX-PP exerts its effect directly.

We next examined the effects of purified rLOX-PP (22) on fibronectin-stimulated cell migration. Parental NF639 cells were pretreated with rLOX-PP (0, 50, and 250 nM) for 24 h, and then subjected to Boyden chamber haptotaxis assays in the presence of 0, 50, and 250 nM rLOX-PP. As shown in Fig. 4C, rLOX-PP treatment resulted in a dose-dependent reduction in migration of NF639 cells (27% at the higher dose), consistent with the data obtained with cells overexpressing LOX-PP (Fig. 4A). Furthermore we examined the effect of rLOX-PP on adhesion structures. NF639 cells were pretreated with rLOX-PP (0 and 250 nM) for 24 h, and then plated on fibronectin-coated chamber slides in the presence of 0 and 250 nM rLOX-PP. After an additional 24 h, cells were stained for filamentous actin (F-actin) and tyrosine phosphorylation. A change in morphology was apparent upon treatment with rLOX-PP. NF639-treated cells displayed a more triangular shape (Fig. 5, upper panels). Moreover, analysis of the high magnification images revealed a profoundly reduced concentration of phosphorylated proteins in focal adhesions (Fig. 5, middle and lower panels), supporting our findings that LOX-PP inhibits fibronectin-stimulated tyrosine phosphorylation of FAK and p130Cas (Figs. 2 and 3).

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**DISCUSSION**

Here we show for the first time that expression of LOX-PP attenuates fibronectin-mediated tyrosine kinase signaling, as determined by reduction in tyrosine-phosphorylated FAK and
p130Cas proteins, and decreased haptotaxis. Integrin clustering leads to autophosphorylation of FAK at Tyr-397, which provides a binding site for the non-receptor tyrosine kinase c-Src, which in turn phosphorylates Tyr-576 and Tyr-577 in the catalytic domain of FAK (28). Ectopic LOX-PP expression in NF639 breast cancer cells is here shown to interfere with the phosphorylation of FAK on Tyr-397 and Tyr-576, and to result in less recruitment of its substrate p130Cas to focal adhesion sites (Fig. 7). Importantly, both ectopically expressed and purified recombinant LOX-PP significantly and selectively reduced fibronectin-mediated cell migration in NF639 cells, and this effect was extended to haptotaxis of two highly migratory human breast cancer cell lines: MDA-MB-231 and Hs578T. Of note, these effects were specific for the ECM protein fibronectin, as no such inhibition was seen on laminin and vitronectin.

Elevated fibronectin levels observed during tumor progression contribute to enhanced signaling of its cognate integrin receptors. Integrins are major adhesion and signaling proteins. Their activation supports cell survival and motility either by directly affecting downstream signaling cascades involving FAK and p130Cas, or by cross-talk with receptor tyrosine kinases. Though many cancer cells display anchorage-independent properties, they do benefit from integrin signaling (29). Moreover, aberrant expression of the fibronectin receptors \( \alpha_5\beta_1 \) and \( \alpha_6\beta_1 \) integrin have been observed in various cancers, including breast cancer (30). Importantly, these integrins are crucial for invasion and migration of tumor cells, which involves physical tethering of cells to the matrix, as well as sending and receiving molecular signals that regulate these processes. Data presented here suggest that LOX-PP specifically alters the fibronectin-integrin interaction, and thus by this
LOX-PP Inhibits Integrin Signaling

FIGURE 7. Model of LOX-PP regulation of FN-stimulated signaling and migration. Integrin engagement on fibronectin leads to recruitment and phosphorylation of FAK, c-Src, and p130^Cas to focal adhesions. Tyrosine-phosphorylated p130^Cas binds the small adapter molecule Crk constituting a molecular switch, which promotes migration. Pro-LOX and LOX-PP are secreted into the extracellular space, and may directly interfere with FN-stimulated activation of its receptors (indicated by a cut line). Arrows with solid lines indicated direct interaction. Arrows with dashed lines indicated indirect interactions.

action it may contribute to the tumor suppressor activity of LOX-PP, which was recently revealed (6, 14, 15).

It is notable that, FAK and p130^Cas proteins, which play crucial roles in integrating pro-migratory signals, are activated and/or overexpressed as well in a variety of human tumors, including breast cancers (31, 32). Moreover, their expression is associated with advanced and aggressive phenotype (33, 34). Thus, the findings of the inhibitory effect of LOX-PP on this pivotal signaling node suggest that further investigation of its therapeutic potential to inhibit invasive cancers should be investigated.

Interestingly, while the LOX gene itself was identified as the Ras recision gene with tumor suppressor activity on Ras-mediated transformation, active LOX enzyme was recently found to promote invasive phenotype in MDA-MB-231 and MCF-7 breast cancer cells ectopically expressing the mature LOX enzyme (27, 35). This apparent paradox can be explained by mapping of the Ras recision activity to the LOX-PP domain (14). Consistent with this bifunctionality, we noted that Pro-LOX and LOX-PP suppressed fibronectin-stimulated cell signaling and migration, while expression of the mature LOX enzyme stimulated migration. The stimulating action of LOX is consistent with findings by Payne et al. (36), who reported that the expression of sequences encoding the LOX enzyme in MCF-7 cells facilitated the activation of FAK (36). Furthermore, the enzymatic activity of LOX has recently been associated with FAK activation in invasive astrocytes (37). Similarly, in our previous study we noted that while LOX-PP reduces NF-κB binding and formation of invasive colonies in Matrigel by NF639 breast cancer cells, LOX induces NF-κB and invasive colony formation and reduces epithelial markers including estrogen receptor α and γ-catenin (6). Overall, these findings suggest LOX-PP and LOX have opposing effects on the migratory phenotype of cancer cells. Consistent with this hypothesis, we found that treatment of NF639-LOX cells with 250 nM rLOX-PP attenuated the observed increase in haptotaxis by LOX enzyme, suggesting that LOX-PP indeed regulates the tumor promoting activity of the mature enzyme (data not shown). It should be noted that unlike natural Pro-LOX, ectopically expressed mature LOX lacking LOX-PP but containing a signal peptide, is not secreted (present study and Ref. 6, 25). These constructs might alter the physiologically relevant range of biological activities observed in these assays. It was reported that a deletion construct containing the signal peptide and retaining the last 30 residues of the pro-peptide still results in the secretion and activation of Pro-LOX (38). By contrast, the complete absence of the LOX-PP domain resulted in no observable secretion of LOX proteins (25). The present study shows that ectopic expression of Pro-LOX, that is secreted normally and extracellularly processed into active LOX and LOX-PP, attenuates integrin signaling and fibronectin-stimulated haptotaxis, suggesting that LOX-PP effects are dominant.

Recently, it has been shown that Pro-LOX interacts directly with fibronectin (17). Moreover, Fogelgren et al. (17) have suggested that fibronectin promotes processing of Pro-LOX and acts as a scaffold for active LOX. It is feasible that the pro-peptide domain of LOX has a regulatory role in this interaction. This idea is supported by our findings that the inhibitory activity of LOX-PP strongly affects fibronectin- and not so much laminin- or vitronectin-stimulated integrin activation. Elevated fibronectin levels have been observed during progression to a more invasive phenotype. This increase in fibronectin may enhance Pro-LOX processing in tumors where it is expressed. By this mechanism it would enhance the enzymatic activity of LOX and might counteract the tumor suppressor function of the LOX-PP. Moreover, it has been demonstrated that LOX-PP plays a crucial role in the deposition of the enzyme onto elastic fibers by interacting with tropoelastin (39). During tumor progression, the microenvironment undergoes significant changes. In particular, dense aggregation of elastic fibers is found in malignant tumors of the breast and lung (40, 41). Whether these changes contribute to the tumor promoting action of the active LOX enzyme has not yet been determined. In summary, the studies described here provide important new information on the mechanisms of action by which LOX-PP functions as an inhibitor of signaling in tumor cells.

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