The Pro-regions of Lysyl Oxidase and Lysyl Oxidase-like 1 Are Required for Deposition onto Elastic Fibers

Laetitia Thomassin, Claudio C. Werneck, Thomas J. Broekelmann, Claudine Gleyzal, Ian K. Hornstra, Robert P. Mecham, and Pascal Sommer

These studies were undertaken to determine how lysyl oxidase (LOX) and lysyl oxidase-like-1 (LOXL) enzymes are targeted to their substrates in the extracellular matrix. Full-length LOX/LOXL and constructs containing just the pro-regions of each enzyme localized to elastic fibers when expressed in cultured cells. However, the LOXL catalytic domain without the pro-region was secreted into the medium but did not associate with matrix. Ligand blot and mammalian two-hybrid assays confirmed an interaction between tropoelastin and the pro-regions of both LOX and LOXL. Immuno-fluorescence studies localized both enzymes to elastin at the earliest stages of elastic fiber assembly. Our results showed that the pro-regions of LOX and LOXL play a significant role in directing the deposition of both enzymes onto elastic fibers by mediating interactions with tropoelastin. These findings confirmed that an important element of substrate recognition lies in the pro-domain region of the molecule and that the pro-form of the enzyme is what initially interacts with the matrix substrate. These results have raised the interesting possibility that sequence differences between the pro-domain of LOX and LOXL account for some of the functional differences observed for the two enzymes.

Production of a mature and functional elastic fiber is a complex process that is only partially understood. Monomers of elastin (tropoelastin) are cross-linked in the extracellular space by one or more members of the lysyl oxidase (LOX) gene family to form an elastin polymer, which is the functional form of the mature protein. Fibrillin-containing microfibrils are thought to play an important role in the assembly process by serving as a scaffold for aligning cross-linking domains within tropoelastin. Recently, several other proteins, such as members of the fibrillin and emilin families, have been suggested to play a role in elastic fiber formation, although their exact function has not yet been determined.

LOs are extracellular copper-requiring enzymes that catalyze the cross-linking of collagen and elastin through oxidative deamination of lysine or hydroxylysine side chains. The resultant allysine residues can then spontaneously condense with vicinal peptidyl aldehydes or with e-amino groups of peptidyl lysines to generate covalent cross-linkages. There are five members of the LO family: lysyl oxidase (LOX) and lysyl oxidase-like 1–4 (LOXL 1–4) (reviewed in Ref. 2). The C-terminal region of all of the LO family members contains the elements required for catalytic activity (the copper binding site, tyrosyl and lysyl residues that contribute to the carbonyl cofactor, and 10 cysteine residues), and the high sequence homology in this region suggests that all family members share a common enzymatic mechanism. The N-terminal regions, in contrast, show the greatest variability in size and sequence.

The genes for LOX and LOXL have a similar exon structure consisting of seven exons, five of which (exons 2–6) are of similar size and encode proteins with 76% amino acid identity (3). Differences between LOX and LOXL reside mainly in sequences coded by exon 1. In LOX, exon 1 codes for a signal peptide, a putative pro-enzyme region, and the beginning of the mature enzyme. In LOXL, this exon is larger than exon 1 in LOX and encodes a proline-rich region. Both LOX and LOXL are enzymatically active after processing from their secreted pro-forms by procollagen C-proteinase (PCP) or mammalian tolloid proteases (4, 5). The exact maturation site of LOXL, however, is still unidentified (4, 6). The pro-peptides of LOX and LOXL have a similar cationic charge profile but have less overall homology than the mature domains of each enzyme (7). LOXL-2, -3, and -4 are the largest family members and have cysteine-rich scavenger receptor-like domains in their N-terminal region that are not found in LOX and LOXL.

The substrate preferences or specificities of the various LO family members are not known. LOX and LOXL are the best studied, and both have been shown in vitro to use tropoelastin as a substrate (4, 8). The ability of both enzymes to cross-link elastin has also been confirmed in vivo by lox (9) and loxl (10) gene targeting underexpression studies resulting in ~60 and ~40%, respectively, decreased desmosine cross-links in lung tissue of deficient mice. The decreased elastin content in LOX knock-out mice results in death shortly after birth due to aortic dissection, cardiovascular dysfunction, and diaphragmatic rupture (9, 11). Ultrastructural analysis shows poorly developed, fragmented, and discontinuous elastic fibers in aorta, lung, and skin. LOXL-null mice, in contrast, have a mild connective tissue phenotype characterized by pelvic laxity in female animals and enlarged pulmonary air spaces that result from decreased elastin content4 (10). In addition, Liu et al. (10) reported that the LOXL-null mice do not deposit normal elastic fibers in the postpartum uterus.

A recent study of LOX and LOXL distribution in the developing mouse (12) found co-localization of both enzymes in the tissues that are

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1 A recipient of studentships from the French government.

2 To whom correspondence should be addressed: Dept. of Cell Biology and Physiology, WA University School of Medicine, Campus Box 8238, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2254; Fax: 314-362-2252; E-mail: bmecham@cellbiology.wustl.edu.

3 The abbreviations used are: LO, lysyl oxidase; LOX, lysyl oxidase; LOXL, lysyl oxidase-like; Jmat, mature region deleted; pro, pro-region deleted; FL, full-length; PCP, pro-collagen C protease; RFL-6, rat fetal lung fibroblasts-6; TE, tropoelastin; PBS, phosphate-buffered saline; ECM, extracellular matrix; Cter, C terminus; AD, activating domain; BD, binding domain.

4 Ian Hornstra, unpublished results.
most affected in the knock-out animals. Heart, lung, and skin, for example, have overlapping localization of LOX and LOXL. Furthermore, both enzymes co-localize to the same elastic fibers of human skin using immunoelectron microscopy (13). These findings suggest that even if LOX and LOXL have the same catalytic activity and are expressed in the same tissues, they appear to not have completely redundant roles in elastic fibers formation and homeostasis. The reason that one enzyme may not compensate for the other in the knock-out animals may relate to differing substrate specificities resulting from substrate interaction determinants outside of the catalytic domain.

The present studies were undertaken to determine how LOX and LOXL are targeted to elastic fibers. A recombinant approach was developed to study the role of each domain of LOX and LOXL in a lung RFL-6 fibroblast model. Direct protein binding assays were also used to characterize the binding region for LOX and LOXL within tropoelastin. Our results showed that the pro-regions of LOX and LOXL play a significant role in directing the deposition of both enzymes onto elastic fibers by mediating interactions with tropoelastin. This has raised the interesting possibility that functional differences between the two enzymes will be manifested when LOX and LOXL are still pro-enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were obtained from Sigma, unless otherwise specified.

**Antibodies**—A polyclonal antibody against human recombinant LOX (anti-LOX238–279) was used as described (13) for LOX immunolocalization and Western blot analysis. For LOXL Western blot analysis, a polyclonal antibody against human recombinant LOXL (anti-LOXL355–417) was used as described (13). A new polyclonal antibody against recombinant mouse LOXL (anti-LOXL204–305) was used for immunolocalization of LOXL. This antibody was prepared by immunizing rabbits with the C-terminal two-thirds (45 kDa, starting with the sequence VYYRG) of the mouse LOXL cDNA cloned into the EcoRI site of pET-28b and purified from a bacterial extract using metal affinity chromatography. Sequence VYYRG was synthesized and cloned in-frame behind the Gal4 binding domain of the pBIND vector. The beginning and the end of each insert are shown in TABLE ONE. Construct integrity was verified by sequencing (GENOME TECHNICAL SERVICES, Meylan France). Sequences were analyzed with the National Center for Biotechnology Information Basic Local Alignment Search Tool software (Applied Biosystems, Foster City, CA). Plasmids were purified using a NucleoBond® AX PC 100 DNA purification kit (MACHEREY-NAGEL). Each vector was expressed in RFL-6 or HeLa cells to confirm the correct size of the translated protein.

**Transient Transfection Constructs and Mutagenesis**—The transfection constructs used for this study were derived from a previously characterized full-length murine cDNA of LOX and from a full-length human cDNA of LOXL (13, 15). Chimeric V5 fusion proteins were inserted in-frame with the C-terminal V5/His tag within the pcDNA4-V5/His vector (Invitrogen). Schematics of the chimeras are shown in Figs. 3 and 4. The LOX constructs that were expressed in this study include: (a) full-length (FL) murine LOX (FL-LOX-V5); (b) LOX with its pro region deleted (Δpro); (Δpro-LOX-V5); and (c) unmutated LOX, with its PCP maturation site deleted (ΔPCP) (ΔPCP site-LOX-V5). These constructs have been characterized previously (16). LOX with its catalytic (i.e. mature) domain deleted (Δmat) (Δmat-LOX-V5) was generated by subcloning a BamHI-Apal fragment of ΔPCP-LOX-V5 into a new pcDNA4-V5/His vector. An expression construct for full-length human LOXL (FL-LOXL-V5) has been described previously (17). Several steps were required to generate a Δpro-LOX-V5 and Δmat-LOXL-V5 plasmid. First, a mutant of the FL-LOXL-V5 vector containing an XhoI site at +910 was generated using PCR primers (5’TCTCGAGCCACGGCTGGGCTGG-3’ and 5’-TACTCGAGCATGGGCGCTGGCCGC-3’). The mutant fragment was then cut using BamHI and XhoI and inserted in-frame before the V5/His sequence of a new pcDNA4-V5/His vector, thereby generating the Δmat-LOXL-V5 plasmid. The Δpro-LOXL-V5 plasmid was generated by cutting the mutant vector with SmaI and XhoI and filling the resultant linearized vector with Klenow fragment followed by self-ligation.

All of the human LO constructs for mammalian two-hybrid assay were cloned in-frame behind the Gal4 binding domain of the pBIND vector (Promega, Madison, WI). A tropoelastin fragment containing exons 19–36 of human TE was cloned in-frame behind the VP16-activating domain of the pACT vector (Promega) to generate pAD-TE-Cter vector. The beginning and the end of each insert are shown in TABLE ONE. Construct integrity was verified by sequencing (GENOME EXPRESS, Meylan France). Sequences were analyzed with the National Center for Biotechnology Information Basic Local Alignment Search Tool software (Applied Biosystems, Foster City, CA). Plasmids were purified using a NucleoBond® AX PC 100 DNA purification kit (MACHEREY-NAGEL). Each vector was expressed in RFL-6 or HeLa cells to confirm the correct size of the translated protein.

**Cell Culture and Media**—The rat fetal lung fibroblast cell line RFL-6 was purchased from the American Type Culture Collection, Manassas, VA (ATCC, CCL-192), and cells were maintained in Ham’s nutrient mixture F-12 supplemented with 2.5 g/liter sodium bicarbonate, 20% Cosmic calf serum (HyClone), 2 mM glutamine (Cellgro, Herndon, VA), 20% fetal calf serum (HyClone), and 2 mM glutamine (Cellgro, Herndon, VA).

**TABLE ONE**

| Description of mammalian 2-hybrid construct insert borders |
|-----------------------------------------------------------|
| Name of the hybrid | Amino Acids | Sequence |
|---------------------|-------------|----------|
| BD-LOX-FL | +18 to +417 | VHCPAAAGQ...CTISPY- |
| BD-LOX-pro | +18 to +164 | VHCPAAAGQ...SNLPPRS |
| BD-LOX-mat | +165 to +417 | GDPNPYPK...CTISPY- |
| BD-LOXL-FL | +40 to +571 | WRLIQWEN...VSAWNKCI |
| BD-LOXL-pro | +40 to +310 | WRLIQWEN...GDPRLKWY |
| BD-LOXL-mat | +310 to +571 | YPPYANPPE...VSAWNKCI |
| AD-TE-Cter | +433 to the end | GVGISPEAQA...GRKRR |

*All the constructs were derived from human cDNAs. The sequence numbering is based on the cDNA.*
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non-essential amino acids (Cellgro), 10 units/ml penicillin, and 10 µg/ml streptomycin (Washington University Tissue Culture Supply Center). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 2 mM glutamine, 10 µg/ml gentamicin.

DNA Transfections—RFL-6 cells were seeded at ~60% confluency onto 6-well plates, some of which contained sterile coverslips. Three days after visual confluency and immediately before transfection, the culture medium was replaced with fresh growth medium without antibiotic, and the cells were transfected with 4 µg of the appropriate plasmid using 10 µl of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cells were then incubated for 48 h before immunostaining or extraction of recombinant proteins.

Protein Extraction, SDS-PAGE, and Western Blot Analysis—To study the secretion of recombinant LOs, medium from 48 h-transfected or untransfected cells was collected. After clearing by centrifugation, 2 ml of supernatant was incubated with 50 µl of nickel-nitrilotriacetic acid agarose (Qiagen) for 45 min at 4 °C. After two washes in 20 ml imidazole, 50 mM NaH2PO4, 300 mM NaCl, the resins were collected, and proteins were eluted by boiling in SDS-PAGE sample buffer containing 50 mM dithiothreitol. Cell layers were lysed using 8M urea in 16 mM Tris-HCl pH 7.5. The lysates were separated by gel electrophoresis using 10% PhastGels (Amersham Biosciences).

Expression and Purification of Tropoelastin and Fragments—A full-length tropoelastin construct, generated from the bovine-C variant, was cloned into the pQE expression system using a BamHI linker in place of the initiator methionine as described previously (18). Subfragments were generated from the full-length construct using restriction enzyme digestions. The exon 1–15 construct was a HindIII fragment and represents amino acid residues 2–274. The exon 15–36 construct begins at the HindIII site and represents amino acid residues 275–747. The residue numbering is based on the translated bovine A splice variant (accession number P04985) beginning with the initial methionine (19). The bovine C variant has exons 13 and 14 spliced out.

Full-length constructs and fragments of recombinant bovine tropoelastin were expressed as His6 fusion proteins with the pQE vectors (Qiagen) in the M-15 strain of E. coli. Expressed proteins were initially purified using nickel chromatography under denaturing conditions using the batch purification method described by Qiagen. Eluted tropoelastin or tropoelastin fragments were dialyzed against 50 mM acetic acid, lyophilized, and then further purified using reverse phase high pressure liquid chromatography on a Vydac 214TP510 C-4 column using a 0–50% of acetonitrile gradient. Peak fractions were dried by rotary evaporation, dissolved in MilliQ water, and then lyophilized.

Purity of the expressed proteins was assessed by SDS-PAGE using 8–25% PhastGels (Amersham Biosciences). In Vitro Translation Product and Recombinant Protein—The V5 plasmids were used as DNA templates for in vitro translation with TnT-coupled reticulocyte lysate systems (Promega) in the presence of [35S]methionine. The translation products were analyzed by 10% SDS-PAGE, analyzed by autoradiography, and used as antigen substrate in ligand blot assay.

Ligand Blot Analysis—TE peptides were run on 10% SDS-PAGE gels under non-reducing conditions and transferred to nitrocellulose as described above. The blots were blocked after transfer with 5% (w/v) nonfat milk and 0.1% Tween 20 in PBS (blocking solution) for 1 h at room temperature. 100 µl of LOX or LOXL generated by in vitro translation were added to the blocking solution, and the blots were incubated for 90 min at 37 °C under gentle shaking. The blots were washed three times with 0.1% PBS-T and washed twice with PBS. Membranes were incubated with a 1:100 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG or horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Biosciences) in blocking buffer for 1 h, washed twice, and detected with the ECL system (Amerham Biosciences). The membranes were incubated with a 1:1000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG or horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Biosciences) in blocking buffer for 1 h, washed twice, and detected with the ECL system (Amerham Biosciences). The membranes were incubated with a 1:1000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG or horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Biosciences) in blocking buffer for 1 h, washed twice, and detected with the ECL system (Amerham Biosciences). The membranes were incubated with a 1:1000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG or horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Biosciences) in blocking buffer for 1 h, washed twice, and detected with the ECL system (Amerham Biosciences).
times in blocking agent and dried. Autoradiography was used to detect bound, radiolabeled proteins generated by in vitro translation.

RESULTS

Endogenous LOX and LOXL Are Targeted to Extracellular Fibers of Lung Fibroblasts—RFL-6 cells were used as a model system to study the synthesis, secretion, and targeting of lysyl oxidase to the extracellular matrix, particularly to elastic fibers. RFL-6 cells are derived from fetal rat lung mesenchyme. They produce LOX (20) and deposit an elaborate fibrillar matrix containing fibrillin-1, fibrillin-2, tropoelastin (21), and other ECM proteins. To characterize the time course of endogenous LOX and LOXL expression, endogenous proteins were extracted from the cell layer 2 days before and 1, 3, 5, and 8 days after confluence. LOX and LOXL were then detected by Western blot analysis using polyclonal LOX228–279 and LOXL355–416 antibodies. LOX was identified in the cell layer as a 50-kDa precursor and a 32-kDa mature form (Fig. 1A). A weaker band was also detected at 48 kDa, which has been described as the prepro-enzyme without glycosylation. LOXL was expressed as a 60-kDa precursor and a mature form at 31 kDa (Fig. 1B). LOX and LOXL expression occurred throughout the culture period, whereas maturation was highest around 3 days of confluency (Fig. 1, A and B) at a time when RFL-6 cells start to produce and assemble extracellular matrix components (21). A band slightly smaller than 200 kDa was detectable after 8 days of confluency and most likely resulted from LOXL polymerization.

To localize LOs in the extracellular matrix, RFL-6 cells were fixed 5 days after confluency and stained with LOX228–279 and LOXL204–393 antibodies. Endogenous LOX and LOXL were both localized to extracellular matrix components. Immunostaining of permeabilized RFL-6 cell cultures showed LOX and LOXL to be distributed intracellularly throughout the endoplasmic reticulum and the Golgi apparatus and in secretory vesicles (data not shown). The enzymes were also detected on fibrous extracellular matrix (Fig. 2, A and B) at a time when RFL-6 produced elastic fibers (21). The specificity of the LOXL204–393 antibody was established by showing immunoreactivity with an appropriately sized band in 6 M urea extracts of wild type but not LOXL knock-out adult mouse heart and lung (Fig. 2C). The specificity of anti-LOX228–279 has been established previously (13).

Recombinant LOX Is Targeted to Elastic Fibers in RFL-6 Extracellular Matrix—To identify which regions of LOX and LOXL interact with elastic fiber proteins, domain-encoding constructs containing antibody epitope tags were expressed in RFL-6 cells transfected 3 days after confluency. Expressed recombinant protein was assessed in medium and in cell layer extracts by immunoblot 48 h after transfection. Protein in the cell layer was visualized by indirect immunofluorescence. No difference in matrix deposition was observed using either the murine or the human LOX-V5/His cDNAs (data not shown). LOX-V5 was detected in the ECM at both 48 h and 72 h after transfection. The distribution of LOX-V5 was similar to that of untagged LOX. Both LOX and LOX-V5 were secreted, exhibited the same matrix distribution, and associated with extracellular fibers (Figs. 2A and 3D). Some of these fibers colocalized with fibronecin (data not shown), whereas others were typical of collagen fibers. Of relevance to this study, however, was the colocalization of both endogenous and recombinant LOX with elastic fibers (Fig. 3F), which establishes RFL-6 cells as a useful model for studying lysyl oxidase expression and interactions with extracellular elastic components.

The Pro-region Is Sufficient for LOX-V5 Deposition onto Elastic Fibers—Western blot analysis with V5 antibody of cell extracts and medium from LOX-V5-transfected cells identified a protein of about 55 kDa in the cell layer that is the appropriate size for the full-length protein with a V5-His tag (Fig. 3, P and Q). The mature form with an appropriate molecular mass of around 36 kDa resulting from cleavage of the pro-domain was found in both the cell layer and the cell culture medium. Because the antibody used for immunostaining recognizes both pro- and mature (i.e. processed) forms of LOX, it was not possible to determine which form of the protein was binding to the elastic fibers. To determine which regions of LOX are required for its association with elastic fibers, mutated or deleted forms of LOX-V5 were generated (Fig. 3) and transfected into 3-days post-confluent RFL-6 cells. Immunofluorescence microscopy and Western blotting of medium and cell layer extracts were then performed to determine whether the expressed proteins associated with elastic fibers.

To determine whether the mature protein alone could bind to elastic fibers, RFL-6 cells were transfected with a LOX construct lacking the pro-region (Δpro-LOX-V5), which was created by deleting amino acids immediately after the signal peptide cleavage site up to the PCP cleavage site (from Pro23 to Ile157). As has been reported previously in myofibroblast-like cells (16), the protein from this construct was only detected inside the cells and not detected in the medium (Fig. 3G). A second construct, mutated at Asp163 in the PCP cleavage site (ΔPCP site-LOX-V5) so that processing of the pro-domain cannot occur, was secreted,
and extensive association with extracellular elastic fibers was observed (Fig. 3L). The 56-kDa recombinant precursor was detected in both the cell layer and the cell culture medium (Fig. 3, P and Q).

Results with the mature and unmaturable LOX constructs indicated that maturation of LOX is not required for enzyme deposition into the matrix and suggested that the pro-region may play a role in efficient extracellular targeting. This possibility was investigated by expressing a LOX construct containing just the pro-region of the protein (ΔH9004mat-LOX-V5), developed by deleting sequence distal to the BMP-1 cleavage site (deletion from Asp163 to the end). The expressed protein had an appropriate molecular mass of 23 kDa and was detected by immunoblot in both the cell layer and the cell culture medium (Fig. 3, P and Q).

Staining of transfected cells with a V5 antibody localized the LOX pro-region protein with elastic fibers in the cell layer (Fig. 3O), confirming that the pro-region mediates the association between LOX and elastic fiber proteins. The results also showed that processing from the pro-form to the mature form of the enzyme is not required for targeting to ECM.

The Putative LOXL Pro-region Is Required for the Association of LOXL with Elastic Fibers—Having shown that the pro-region of LOX was sufficient to target LOX to elastic fibers, we next determined whether a similar functional domain could be identified in LOXL. Expression of the full-length LOXL-V5 construct in RFL-6 cells resulted in localization of the recombinant protein to elastic fibers, as was observed for the endogenous protein (Fig. 4, A–C). We also noticed that LOXL-V5 co-localized with elastin globules (Fig. 4J) that have been shown to coalesce to form fibers (21–23). In general, the immunofluorescent staining pattern obtained with LOXL-V5 was similar to what was observed with LOX-V5 and endogenous LOX and LOXL proteins in our RFL-6 model.

To determine whether the LOXL pro-region has a role in targeting LOXL to elastic fibers, LOXL mutants were generated and tested in RFL-6 cells, as was described above for LOX. Because the exact pro-region processing site of LOXL is still unknown (4, 6), we used a putative PCP cleavage site (4) at position Gly303–Asp304 (residue numbers based on the human sequence) to generate a LOXL expression construct lacking the putative pro-region (Δpro-LOXL-V5). The lack of the putative
pro-region of LOXL (amino acids 31–303) did not prevent secretion of the LOXL mutant, as Δpro-LOXL-V5 was detected at high levels in RFL-6 cell-conditioned medium (Fig. 4, middle column). The absence of this region, however, completely abolished the deposition of LOXL into the RFL-6 cell ECM (Fig. 4, D–F). Importantly, cells expressing the pro-region alone (amino acids 1–303, Δmat-LOXL-V5) directed the recombinant protein to matrix elastic fibers. These findings, together with similar results for LOX, suggested that the N-terminal regions of both enzymes contain important trafficking signals for directing the enzyme association with elastic fibers in the extracellular matrix.

The LOX and LOXL Pro-regions Directly Interact with Tropoelastin—To elucidate the interactive sites for LOX and LOXL in tropoelastin, interaction studies were performed using both mammalian two-hybrid and ligand blotting assays. Ligand blots were performed using LO domains (Δmat-LOX-V5, Δpro-LOX-V5, Δmat-LOXL-V5, Δpro-LOXL-V5) generated by in vitro translation to probe bacterially expressed tropoelastin fragments (exons 1–15 and 15–36) that were bound to the solid phase. The results showed a direct interaction between the LOX and LOXL pro-regions and the tropoelastin fragment containing the C terminus (exons 15–36) of the molecule (Fig. 5). Both LOX and LOXL mature regions were reactive with this region of tropoelastin. No interaction with the N-terminal fragment of tropoelastin (exons 1–15) was observed with any of the LO proteins.

Mammalian two-hybrid assays were then used to confirm the interaction observed in ligand blots between LO and the C-terminal region of tropoelastin. For these studies, a subfragment of the C-terminal region of human tropoelastin encompassing exons 19–36 was inserted in-frame behind the Gal4-activating domain of the pACT vector (pAD-TE-Cter). Regions from LOX and LOXL were inserted in-frame behind the Gal4 binding domain (BD-LO) of the pBIND vector. Results from

FIGURE 4. Deletion of putative proregion decreased, but did not completely inhibit, LOXL-V5 deposition on elastic fibers. RFL-6 cells transfected with human FL, Δpro-, or Δmat-LOXL-V5 expression constructs were stained with antibodies against the V5 epitope tag (left column) and with the tropoelastin N6–17 antibody (middle column). A merge of the two labels is presented in the right column. LOXL-V5 was deposited onto elastic fibers (A–C), whereas Δpro-LOXL-V5 was secreted but did not associate with the extracellular matrix (D–F). The deposition of Δmat-LOXL-V5 was restricted to fibers close to transfected cells (G–I). Images were acquired with a ×40 objective at a final optical magnification of ×400. The localization of recombinant LOXL-V5 to elastic globules is shown in J, which is a merged image of LOXL-V5 and tropoelastin staining. It was acquired using a ×100 objective. Scale bars represent 50 μm. The expression of all of the recombinant proteins was confirmed by immunoblot analysis of the cell culture medium and cell layer extracts using the V5 antibody (K and L). The apparent molecular mass was estimated from the migration of molecular mass markers.
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RFL-6 cells provide a useful model system to study collagen and elastin assembly in vitro. These cells secrete and organize an abundant extracellular matrix that includes an extensive elastic fiber network. Similar to what has been reported in other studies (10, 13), we were able to show colocalization of LOX and LOXL with elastic fibers produced by the cultured cells. The time course of LO secretion was similar to the secretion and organization of ECM proteins by RFL-6 cells, suggesting that LO production is regulated similarly to other matrix proteins.

The association of LOs with elastic fibers in cultured cells is consistent with our ligand binding studies demonstrating a direct interaction between tropoelastin and both LOX and LOXL. In mapping the interaction sites between LOs and tropoelastin, it is interesting that both enzymes bound to the C-terminal (exons 16–36) but not N-terminal (exons 1–15) region of the tropoelastin molecule. Although cross-linking domains can be found in both the front and the back halves of the protein, those in the front half are predominantly the KP-type sequences (lysines surrounded by proline residues), whereas those in 16–36 are KA-type (lysines embedded in polyalanine sequences). Many of the cross-links in elastin arise through the condensation of one oxidized and one unoxidized lysine side chain. Previous studies by Brown-Augsburger et al. (24) showed that lysine residues within exon 10, a KP domain, contribute the amino group to the bifunctional cross-link lysinoornelurine, suggesting that this region of the protein is not acted upon by lysyl oxidase. Interestingly, Mithieux et al. (25) found that the region spanning exons 2–15 of human elastin was also enriched in lysinoornelurine cross-links when the full-length protein was used as a substrate for a yeast form of lysyl oxidase. Although this latter study did not identify the donor of the amino group in the cross-link, the data are consistent with those of Brown-Augsburger et al. (24) in suggesting that the amino group is donated by lysines in KP sequences. Our finding that LO did not interact with the tropoelastin 1–15 fragment would explain why lysine residues within this region remain unoxidized and provides insight into the preferred recognition sequences of the enzyme.

It is also interesting that the mature forms of LOX and LOXL (i.e. catalytic portion without the propeptide region) showed binding to the C-terminal half of tropoelastin (exons 16–36) in the ligand blots but little to no binding to the shorter construct (exons 19–36) used in the mammalian two-hybrid assay. It should also be noted that the mammalian two-hybrid assay is not an efficient technique to detect interactions between an enzyme and its substrate because the interaction can be too fast and unstable to allow the transcription of the reporter gene. This would be consistent with the mature region having catalytic activity against the substrate but lacking the stable interaction imparted by the propeptide domain.

There is ample evidence that the mature form of LOs is catalytically active against collagen and elastin substrates when incubated together in vitro (4, 26–28). Our findings, however, suggested that the pro-forms of LOX and LOXL initially target the enzymes to the appropriate matrix substrate. In mammalian two-hybrid and ligand blot assays, all of the constructs containing the pro-regions of the molecule interacted directly with tropoelastin. These same pro-region constructs were found to co-localize to extracellular elastic fibers when expressed in RFL-6 cells. In contrast, the mutant Δ-proLOXL-V5 construct, containing the catalytic domain but lacking a majority of the putative pro-region, was secreted by RFL-6 cells into the medium and not found in the ECM. It should be noted that the mature region of LOX lacking the pro-domain was not secreted when expressed in RFL-6 cells. However, given the high sequence homology (80%) between the catalytic domains of LOX and LOXL, it is likely that the matrix binding properties of this region will be similar for both enzymes.

FIGURE 6. Interactions of LOX pro-region and LOXL N-terminal region with tropoelastin C-terminal domain are demonstrated by mammalian two-hybrid assay. Exons 19–36 of tropoelastin and FL or partial LOX and LOXL cDNAs were subcloned into the mammalian expression vector pACT and pBIND, respectively, as described under “Experimental Procedures.” A ratio of relative light units larger than 1 is indicative of a positive interaction. The interaction sites between LOX or LOXL pro-regions (LOX pro or LOXL pro), and the regions of tropoelastin encoded by exons 19–36 were also positive. Constructs containing only the LOX or LOXL mature forms (LOX mat or LOXL mat) reacted weakly with exons 19–36 of tropoelastin.

the assay are shown in Fig. 6. The chimeric proteins corresponding to complete LOX and LOXL interacted with the AD-TE-Cter protein. These interactions were again observed with the pro-regions of LOX and LOXL. In contrast to what was observed in the ligand blots, there was no detectable interaction between the tropoelastin construct and the mature form of LOX and only a weak interaction with the mature form of LOX. This difference in reactivity may be due to the shorter construct used in the two-hybrid assays.

DISCUSSION

Elastin and collagen function in the extracellular matrix as cross-linked polymers. The importance of the cross-linking process is evident from the tissue failure that occurs when cross-linking of either of these two molecules is incomplete (9–11). It has long been known that cross-linking is catalyzed by lysyl oxidase and the catalytic mechanism, required cofactors, and enzymatic structural requirements have been described (7). For many years, it was thought that one enzyme was responsible for all of the cross-linking of collagen, elastin, and perhaps other proteins. With the identification of multiple lysyl oxidase-like proteins, however, new questions have arisen about the overall biology of this important enzyme family. In this report, we have shown that two members of the lysyl oxidase family, LOX and LOXL, interact with elastic fibers and with tropoelastin through sequences in the pro-region of the secreted pro-enzyme. These results suggested that the pro-region of the enzyme is critical not only in ensuring proper enzyme activation but in identifying the appropriate substrate that is to be acted upon by the enzyme.

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Together, these findings confirmed that an important element of substrate recognition is determined by the pro-domain regions of these molecules and that the pro-forms of the enzymes are what initially interact with the matrix substrate. Targeting the pro-enzyme to the ECM instead of the mature form would offer the advantage of keeping the enzyme in a latent state until the proper substrate is encountered. The pro-region could also be important in regulating the solubility properties of the enzyme. LOX and LOXL are notoriously difficult to extract from the extracellular matrix without the use of strong denaturants or chaotropic reagents. It is likely that cleavage of the pro-region not only alters the activity of the catalytic domain but might also alter the physical properties of the mature form of the enzyme so that it does not easily dissociate from the substrate that it is designed to modify. This could explain the difficulty in extracting the enzymes from tissues.

The association of the pro-enzyme with extracellular substrates raises some intriguing possibilities in terms of LO activation and storage in the ECM. In matrix-producing cells, it is likely that enzyme activation occurs at the cell surface through the action of cell-associated proteases such as PCP (5). LO could then participate in the early cell-associated stages of collagen and elastin polymerization. It is also possible that pro-forms of the enzymes bind onto the growing collagen and elastin fibers, where they can remain in a latent state until activated at subsequent stages of fiber assembly. In the specific case of elastic fiber formation, Kozel et al. (23) have identified two stages of elastic fiber assembly: one that occurs on the cell surface and one that occurs away from the cell. In the first stage (microassembly), tropoelastin molecules self-associate and cross-link to form small globules on the cell surface. These globules eventually leave the cell and coalesce into fibers in the extracellular space (macroassembly). Cross-linking of elastin must occur at both stages (tropoelastin monomer to tropoelastin monomer in the first stage and globule to globule in the second) to establish a functional fiber. Consistent with this requirement, our studies demonstrated that mature and pro-regions of LOX and LOXL can be localized to both globules and more mature fibers in RFL-6 cell ECM. The presence of latent pro-LO within the globules could be an important source of new enzyme at later stages of globular fusion during the macroassembly stage. This "storage" function is also consistent with the observation that LOXL pro-enzyme can be readily extracted from tissues (4, 12).

The active motifs within the pro-regions of LOX and LOXL that direct their association with ECM are not known. These enzymes belong to a family of five isoforms that mainly differ in their pro-region sequences. The pro-regions of LOX and LOXL are different from each other as well as different from the three other LO isoforms that have closely related pro-regions (2). Although the pro-region PCP cleavage site in LOX has been precisely mapped to Asp1163, cleavage of the pro-region of LOXL has been reported to occur with multiple proteases at multiple sites (4). Despite these differences, it is interesting that the two pro-regions display functional similarity in terms of their ability to bind to elastic fibers. Further work is required to determine whether and how they might mediate the interaction of LOX or LOXL with their other known substrates.

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