A Tissue-specific Variant of the Human Lysyl Oxidase-like Protein 3 (LOXL3) Functions as an Amine Oxidase with Substrate Specificity

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Received for publication, February 1, 2006, and in revised form, August 29, 2006. Published, JBC Papers in Press, October 3, 2006, DOI 10.1074/jbc.M600977200

The Journal of Biological Chemistry 2006 281:37282-37290

The human lysyl oxidase-like 3 (LOXL3) encodes a member of the emerging family of lysyl oxidase (LOX) that functions as a copper-dependent amine oxidase. The LOXL3 protein contains four scavenger receptor cysteine-rich domains in the N terminus in addition to the C-terminal characteristic domains of the LOX family, such as a copper binding domain, a cytokine receptor-like domain and residues for the lysyl-tyrosyl quinone cofactor. Using BLASTN searches, we identified a domain and residues for the lysyl-tyrosyl quinone cofactor. Using BLASTN searches, we identified a domain and residues for the lysyl-tyrosyl quinone cofactor. Using BLASTN searches, we identified a domain and residues for the lysyl-tyrosyl quinone cofactor. Using BLASTN searches, we identified a domain and residues for the lysyl-tyrosyl quinone cofactor.

The human lysyl oxidase-like 3 (LOXL3) functions as an amine oxidase with extracellular matrix proteins, such as collagen and elastin (1). LOX oxidizes the epsilon amino groups of peptide lysines to reactive aldehydes. The resulting aldehyde groups then undergo spontaneous condensation with unreacted epsilon amino groups or neighboring aldehyde groups, converting collagen or elastin monomers into insoluble fibers (2, 3). The LOX-mediated cross-linkage of collagen or elastin monomers is an essential step for the biogenesis of fibrillar extracellular matrix in most tissues.

Recent molecular cloning has revealed the existence of a human LOX family consisting of the five paralogues LOX, LOXL1, LOXL2, LOXL3, and LOXL4 (4–8). Each LOX parologue contains a copper-binding domain, residues for lysyl-tyrosyl quinone (LTQ) and a cytokine receptor-like (CRL) domain in the carboxyl (C) termini. The amino (N)-terminal regions of the LOX family members, in contrast, show little sequence homology except that LOXL2, LOXL3, and LOXL4 contain four repeated copies of scavenger receptor cysteine-rich (SRCR) domains in their N termini. The SRCR domains, known to mediate the protein-protein interactions for cell adhesion and cell signaling, are found either on the cell surface proteins or secreted proteins (9, 10). The presence of SRCR domains within LOXL2, LOXL3, and LOXL4 indicates that these three paralogues probably have different functions from LOX and LOXL.

Several new functions, such as tumor suppression, cellular senescence, and chemotaxis, have been attributed to LOX (11–13). The importance of LOX has been further emphasized by the findings that impaired LOX activity contributes to a number of different diseases including atherosclerosis, aortic aneurysms, pulmonary or hepatic fibrosis, cutis laxa, and hypertrophic scars (14–18). However, it has yet to be determined how altered LOX activity contributes to the pathogenesis of these diverse disorders. The presence of LOX family proteins, each containing the functional motifs to be an active amine oxidase, suggests that differential regulation of the LOX paralogues is probably responsible for the diverse functions currently attributed to the single enzyme LOX.

In an attempt to identify more human LOX paralogues, we searched the expressed sequence tag (EST) databases using the BLAST program. We report a LOXL3 variant LOXL3-sv1 that shows distinct exon-intron structure and tissue specificity from LOXL3. LOXL3-sv1 consists of 10 exons, lacking exons 1, 2, 3, and 5 of LOXL3. Further, this novel variant functions as an amine oxidase with distinct tissue and substrate specificities from one another.

Lysyl oxidase (LOX), a copper-dependent amine oxidase, is responsible for the development of lysine-derived cross-links in extracellular matrix proteins, such as collagen and elastin (1). LOX oxidizes the epsilon amino groups of peptide lysines to reactive aldehydes. The resulting aldehyde groups then undergo spontaneous condensation with unreacted epsilon amino groups or neighboring aldehyde groups, converting collagen or elastin monomers into insoluble fibers (2, 3). The LOX-mediated cross-linkage of collagen or elastin monomers is an essential step for the biogenesis of fibrillar extracellular matrix in most tissues.

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active amine oxidase toward elastin and different types of collagens with variable activities. All the amine oxidase activities of LOXL3-sv1 are sensitive to \( \beta \)-aminopropionitrile (BAPN), an irreversible inhibitor of LOX.

**EXPERIMENTAL PROCEDURES**

**EST Data Base Search**—The human LOXL3 cDNA sequence was used to search the human EST data base by the BLASTN program. EST clones showing significant sequence homology to human LOXL3 were purchased from Open Biosystems and were completely sequenced using an ABI 310 automated DNA sequencer.

**Analysis of the Transcription Initiation Site**—Approximately 2 \( \mu \)g of human testis total RNA (Clontech) was reverse-transcribed using 1 pmol of a random primer (Promega) and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. The reverse-transcribed cDNA was then amplified in 30 PCR cycles (94 °C for 30 s, 54–58 °C for 30 s, and 72 °C for 30 s) with a pre-denaturation at 94 °C for 4 min and a final extension at 72 °C for 5 min, using Taq polymerase (Takara). In each PCR analysis, a forward primer 5’-ctctttttgaaagtctcccc-3’ (F1), 5’-aagagagtcacccaggccg-3’ (F2), 5’-caagaggcttccaaag-3’ (F3), or 5’-gatttttctggctgt-3’ (F4) was used with a common reverse primer 5’-acaagggcgggtcagta-3’ (R1). The PCR amplicons were subjected to electrophoresis on 8% polyacrylamide gel.

**RT-PCR Analysis in Human Tissues**—Human Multiple Tissue cDNA Panels (Clontech) were PCR-amplified with primers specific to LOXL3 and LOXL3-sv1 using Taq polymerase (Takara). The LOXL3-specific primers were 5’-aagagagtcacccaggccg-3’ and 5’-ctctttttgaaagtctcccc-3’, and the LOXL3-sv1-specific primers were 5’-agaagagtcacccaggccg-3’ and 5’-gatttttctggctgt-3’. The LOXL3-specific primers were designed to amplify the region corresponding to nucleotide positions 2471–2938 of \( \text{LOXL3} \), respectively. The PCR amplicons were subjected to electrophoresis on 8% polyacrylamide gel.

**Expression and Purification of the LOXL3 and LOXL3-sv1 Proteins**—The LOXL3 and LOXL3-sv1 cDNAs were RT-PCR-amplified from human testis total RNA (Clontech) using Superscript II Reverse Transcriptase (Invitrogen) and Pfu turbo polymerase (Stratagene) according to the manufacturer’s instructions. The recombinant proteins LOXL3-p1, LOXL3-p2, and LOXL3-sv1 were designed to start the open-reading frame at Arg2, Leu233, and Met362 of \( \text{LOXL3} \), respectively. The sequences of oligonucleotide primers used for construction of the expression plasmids are available upon request. A unique restriction site, either NotI or HindIII, was introduced in each primer for convenient subcloning. The PCR-amplified DNA fragments were gel-purified and then ligated into pET21b (Novagen) at the NheI and HindIII restriction sites in-frame with the C-terminal hexahistidine tag. The resulting expression constructs were confirmed to contain the desired sequences by DNA sequencing analysis using a Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer’s instructions. The Escherichia coli strain BL21(DE3) (Novagen) was used for transformation of the pET21-derived expression constructs. The recombinant proteins were expressed, purified, and refolded into an enzymatically active form as previously reported for other LOX family proteins (19).

**Amine Oxidase Assays**—The amine oxidase activity of the LOXL3 and LOXL3-sv1 recombinant proteins were assessed using a peroxidase-coupled fluorometric assay with the Amplex red hydrogen peroxide assay kit (Molecular Probes) as previously described (20). Each reaction contained 10 \( \mu \)g of the LOXL3 or LOXL3-sv1 protein and 2 \( \mu \)M of a substrate. Elastin from bovine neck ligament, collagen type I from calf skin, collagen type II from the chicken sternum cartilage, collagen type III from calf skin, and collagen types IV, VI, VIII, and X from human placenta were used as substrates, all purchased from Sigma. Parallel assays were performed in the absence or presence of 1 \( \mu \)M BAPN. Fluorescence was measured using a fluorescence spectrophotometer (Molecular Devices) with excitation and emission wavelengths of 571 nm and 585 nm. All transfections were repeated in quadruplicate, and the assay results were expressed as the fluorescence increase over that of BAPN controls.

**Western blot Analysis**—A polyclonal antibody of LOXL3-sv1 was generated by immunizing rabbits with the purified LOXL3-sv1 protein. Rabbits were injected intramuscularly with 300–400 \( \mu \)g of the purified protein in buffer containing 6 \( \mu \)M urea, 250 \( \mu \)M imidazole and 10 \( \mu \)M K2HPO4 on days 0, 14, and 21. After the last injection, the rabbits were bled on days 7 and 14, and then antibodies were tested by ELISA assay. Antibodies were purified using a protein A-agarose kit (KPL) according to the manufacturer’s instructions.

**Cellular Localization of the LOXL3-sv1 Protein**—To construct an expression construct of LOXL3-sv1 in mammalian cells, the full coding sequence of LOXL3-sv1 was PCR-amplified from the EST clone BQ424248 using Pfu Turbo polymerase (Stratagene) according to the manufacturer’s instructions. The forward primer 5’-ctctttttgaaagtctcccc-3’ contained a unique EcoRI site, and the reverse primer 5’-acaagggcgggtcagta-3’.
A Tissue-specific Variant of Human LOXL3

A Gene Structure

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complex 1 2 3 4 5 6 7 8 9 10 11 12 13 14
LOXL3
LOXL3-sv1
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B mRNA Structure

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1 2 3 4 5 6 7 8 9 10 11 12 13 14
LOXL3
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FIGURE 1. Exon-intron and mRNA structures. A, schematic diagrams of the exon-intron structure of LOXL3 and LOXL3-sv1. Exons are shown as gray boxes, introns as bold lines. In LOXL3-sv1, the hatched boxes indicate the regions found only in the LOXL3-sv1 cDNA but not in LOXL3. B, schematic diagrams of the mRNA structure of LOXL3 and LOXL3-sv1. The gray boxes indicate the region corresponding to each exon. In the LOXL3-sv1 mRNA, the left and right hatched boxes indicate the 80-bp sequence corresponding to intron 3 of LOXL3 and the 561-bp sequence corresponding to the 3′-proximal genomic region of exon 14, respectively. The translation initiation sites are indicated by an arrow in each mRNA variant.

FIGURE 2. RT-PCR analysis for determination of the transcription initiation site for LOXL3-sv1. A, a schematic diagram of the regions from which the RT-PCR primers were derived. The forward primers F1, F2, F3, and F4 were derived from the intronic region between exons 3 and 4. The common reverse primer R1 was derived from exon 4. B, in RT-PCR analysis with the F3 and F4 primers, expected sizes of amplicons, 130- and 82-bp bands, respectively, were detected. The F1 and F2 primers did not produce any detectable bands, possibly indicating that the transcription initiation site is located in the region between the F2 and F3 primers.

ggtgct-3′ contained a unique Sall site for convenient subcloning. The PCR-amplified DNA fragments were then ligated into pFLAG-CMV-5b (Sigma) in-frame with the C-terminal FLAG tag. The resulting plasmid pFLAG-LOXL3-sv1 was transiently transfected into HEK 293 cells. HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml of penicillin. At post-transfection of 36 h, the cells were washed with Dulbecco’s phosphate-buffered saline and then fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min and then incubated in phosphate-buffered saline with 1% bovine serum albumin for 30 min at room temperature. The cells were stained for 90 min using a 1:200 dilution of mouse monoclonal anti-FLAG (Sigma) in the blocking buffer. The monolayers were then incubated with a 1:200 dilution of fluorescein isothiocyanate monoclonal rat anti-mouse IgG1 (Zymed Laboratories Inc.) in the blocking buffer for 1 h at room temperature before final washing and examination. Nuclei were stained with propidium iodide (1:1000) (Molecular Probes). Confocal microscopic analysis was performed on a Zeiss LSM510 laser-scanning microscope.

Promoter Analysis—For construction of reporter plasmids, the 5′-flanking regions of exons 1 and 4 were separately PCR-amplified from human genomic DNA using Taq polymerase (Takara). For promoter assays of LOXL3, a 1378-bp fragment from nucleotide positions −1 to −1378 was amplified. For promoter assays of LOXL3-sv1, a series of fragments from the 5′-flanking region of exon 4 were PCR-amplified. The sequences of oligonucleotide primers are available upon request. The PCR primers contained either a KpnI or BglII restriction site for convenient subcloning. The PCR-amplified genomic DNA fragments were ligated into a promoter-less luciferase reporter plasmid pGL3-basic (Promega) at KpnI and BglII restriction sites. For promoter assays, HEK 293 cells and primarily-cultured human skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml of penicillin. The cells were plated at a density of 4.5 × 104 cells/well on a 6-well plate 1 day prior to transfection. A total of 2 μg of plasmid DNA containing either a LOXL3 or LOXL3-sv1 promoter construct was co-transfected with 1 μg of the pRSV-LacZ vector expressing the β-galactosidase gene into the cultured cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The pGL3-Basic and pGL3-SV40 plasmids were also used as controls. The cells were harvested 36 h after transfection in a lysis buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride. Luciferase activities of the reporter constructs were determined using a luciferase assay system (Promega), and β-galactosidase assays were performed with a β-galactosidase assay system (Promega). The luciferase activity of each construct was normalized for differences in transfection efficiency based on the results of the β-galactosidase assays. All transfections were repeated in quadruplicate, and the results were expressed as the mean ± S.D.

RESULTS

Exon-Intron Structure of LOXL3-sv1—Searches of the human EST databases for the sequences similar to those of the LOX family genes resulted in identification of five ESTs showing alternative exon-intron splice patterns of LOXL3. Three ESTs (BE731785, BE302919, and BG479178) corresponded to a
previously reported splice variant that lacks exons 5 and 8 of the 14 exons of LOXL3 (21). Two of the ESTs (BE302955 and BQ424258) were novel variants of LOXL3 lacking the sequence corresponding to exon 5. Because only partial sequences of ESTs are, in general, available in the databases, we performed complete sequencing of the ESTs from commercial sources. Complete sequencing of BQ424248 revealed that this novel variant (LOXL3-sv1) consisted of 10 exons, lacking exons 1, 2, 3, and 5 of LOXL3 (Fig. 1A). At the 5′-end, of particular interest, the LOXL3-sv1 cDNA contained an additional 80-bp sequence from the 5′-flanking intronic region of exon 4. At the 3′-end, the LOXL3-sv1 cDNA contained an additional 561 bp sequence corresponding to the 3′-proximal genomic region of exon 14 (Fig. 1B).

To further define the transcription initiation site of LOXL3-sv1, RT-PCR analysis was performed with primers derived from poly(A)⁺ RNA from the human testis (Fig. 2A). The most 5′-end primer that produced an expected size of DNA was the F3 primer derived from the sequence at positions +4,273 to +4,293 (Fig. 2B). The primers derived from the 5′-upstream region of position +4,273 where corresponds to the 5′-end of EST BQ424248 did not produce any detectable bands in the RT-PCR analysis (Fig. 2B), indicating that the probable transcription initiation site of LOXL3-sv1 is located in the region between positions +4182 and +4273.

The exon-intron structure analysis revealed that the LOXL3-sv1 mRNA was at least 2,993 bp in length, encoding a 392 amino acid-long polypeptide with a calculated molecular mass of 44 kDa. The 5′-UTR of the LOXL3-sv1 mRNA was at least 466 bp in length, the coding region was 1,179 bp, and the 3′-UTR was 1,348 bp. The methionine residue at codon 362 in LOXL3 was used as the initiation codon in the deduced amino acid sequence of LOXL3-sv1. The LOXL3-sv1 polypeptide did not contain the SRCR domains 1, 2, and 3 of LOXL3, but did include the characteristic C-terminal domains of the LOX family, including the copper binding domain, the residues for the LTQ cofactor, and the CRL domain (Fig. 3).

RT-PCR Analysis—To assess the expression of LOXL3-sv1 and LOXL3 in human tissues, RT-PCR analysis was performed with forward and reverse primers for the LOXL3-sv1-specific amplicon were derived from the extended 3′-UTR sequence present only in the LOXL3-sv1 mRNA. The PCR primers for the LOXL3-specific amplicon were derived from the sequences of exons 4 and 5, respectively. The LOXL3-specific band was more predominantly detected in the heart, placenta, lung, and small intestine, while the LOXL3-sv1-specific band was more highly detected in the kidney, pancreas, spleen, and thymus (Fig. 4), indicating distinct tissue specificity between LOXL3 and LOXL3-sv1.

Amine Oxidase Activity of LOXL3 and LOXL3-sv1—In an effort to express and purify enzymatically active forms of LOXL3 and LOXL3-sv1, we constructed a series of expression plasmids by subcloning variable lengths of LOXL3 cDNAs into an E. coli expression plasmid pET21b (Fig. 5A). Upon induction
with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C, the hexahistidine-tagged recombinant proteins, except for LOXL3-p1, were expressed at high levels from the expression constructs. Fractionation of the cell lysates into different cellular compartments, such as cytoplasmic extracts, periplasmic extracts and inclusion body fractions, revealed that most of the recombinant proteins were expressed within inclusion bodies. The insoluble recombinant proteins were denatured by urea during purification and were, subsequently, refolded by stepwise dialysis in the presence of N-lauroylsarcosinate and Cu²⁺. The apparent sizes of the expressed recombinant proteins were in good agreement with the deduced molecular mass; 60 kDa for LOXL3-p2 and 46 kDa for LOXL3-sv1 (Fig. 5B). These recombinant proteins were determined to be more than 95% pure on SDS-PAGE gel (Fig. 5B). However, it was not clear why the LOXL3-p1 construct did not show any detectable expression, but it might be because of the presence of rare codons, such as CCC (Pro¹¹, Pro⁶⁰, Pro¹⁷³, Pro⁸¹, Pro¹⁸³, and Pro²²⁰) and AGG (Arg¹⁵¹, Arg¹⁹⁶, and Arg²²⁶), in the N terminus of LOXL3, which are infrequently used in E. coli. Clusters of rare codons were shown to cause translation errors and reduction of the expression level in E. coli (21).

The refolded recombinant LOXL3 and LOXL3-sv1 proteins were assessed for amine oxidase activity toward collagen and elastin using a peroxidase-coupled fluorometric assay. Elastin and collagen types I, II, III, IV, VI, VIII, and X were tested as substrates for the amine oxidase assays. The LOXL3 and LOXL3-sv1 proteins showed significant levels of amine oxidase activity toward all the tested substrates in a BAPN-sensitive manner. In amine oxidase activity, both LOXL3 and LOXL3-sv1 showed a similar pattern of substrate specificity toward the tested substrates, both presenting higher activities toward collagen types IV, X, and I and lower activities toward collagen type VI (Fig. 5C). However, subtle differences in the substrate specificity were also noticed. LOXL3 showed the highest activity toward collagen type VIII, while LOXL3-sv1 presented the highest activity toward collagen type IV (Fig. 5C). These results indicate that both LOXL3 and LOXL3-sv1 function as amine oxidases with similar but different substrate specificity.

Previously, we showed that serial deletion of the N-terminal SRCR domains did not affect the amine oxidase activity in LOXL4, a close parologue of LOXL3 (19). The conserved C-terminal domains of LOXL4 were sufficient to confer the amine oxidase function on LOXL4, though an artificial substrate benzylamine was used for the assays (19). Our current results with LOXL3-sv1 further confirm that truncation or deletion of the SRCR domains does not affect the amine oxidase function of the LOX family proteins toward even physiological substrates of LOX, such as collagen and elastin.

**Western Blot Analysis**—The predicted length of the polypeptide encoded by LOXL3-sv1 was 392 amino acids with a deduced molecular mass of 44 kDa. In an attempt to determine the expression of LOXL3-sv1 at the protein level in vivo, we generated a polyclonal antibody against the purified LOXL3-sv1 protein, which reacted to both the purified recombinant LOXL3 and LOXL3-sv1 proteins in vitro (data not shown). Human testis, placenta, lung, and colon tissues were tested for Western blot analysis. A band with an apparent molecular mass of 44 kDa was detected, though at variable levels, in all the tested human tissues, indicating that LOXL3-sv1 is probably
expressed at the protein level in vivo (Fig. 6). In the lung tissue, however, the LOXL3-sv1 protein was detected in a much lesser extent (Fig. 6). These results are comparable with the RT-PCR results (Fig. 5) that also showed a moderate level of the LOXL3-sv1 expression in the testis, placenta, and colon but almost no expression of LOXL3-sv1 in the lung.

The full-length form of LOXL3 with an expected molecular mass of 83 kDa was not detected in any tissues tested. Instead, a smaller band with an apparent molecular mass of 40 kDa was detected only in the colon (Fig. 6). No apparent bands were seen smaller band with an apparent molecular mass of 67 kDa was detected in the colon and placental tissues (Fig. 6). Additionally, a band with an apparent molecular mass of 83 kDa was not detected in any tissues tested. Instead, a

**Promoter Analysis**—To establish whether the 5′-flanking region of exon 4 of **LOXL3** is sufficient for transcriptional initiation of **LOXL3-sv1**, we generated a series of reporter constructs containing different portions of the 5′-proximal intronic region of exon 4 (Fig. 8A).

The 5′-flanking region of exon 1 was also tested for promoter activity. The promoter activity was monitored by transient transfection in HEK 293 cells and primarily cultured human skin fibroblasts. The pGL3-SV plasmid containing the strong T-antigen promoter of SV40 was used as a positive control, whereas a promoter-less plasmid pGL3-Basic was used as a negative control. The luciferase activity of each construct was expressed as a ratio to that of the promoter-less control. In HEK 293 cells, the P1 construct containing a 1,378 bp fragment of the 5′-proximal region of exon 1 showed ~3 times higher luciferase activity than the P2 construct containing a 1,383 bp fragment from the 5′-flanking region of exon 4 (Fig. 8B). In contrast, the P2 construct showed higher luciferase activity than the P1 construct in human skin fibroblast cells (Fig. 8B), implying differential activation of these two promoters in different cell types. Of particular interest, the P3 construct that lacks the 277-bp sequence corresponding to the 5′-end of the P2 construct showed higher luciferase activity than the P2 construct in both HEK 293 and skin fibroblast cells, indicating possible existence of a transcription repressor element in the deleted region. The P4 construct containing a 344-bp fragment from the 5′-proximity of the P3 construct presented only slightly higher luciferase activity than the P2 construct in different portions of the 5′-flanking region, we generated a series of reporter constructs containing different portions of the 5′-proximal intronic region of exon 4 (Fig. 8A).
binding sites including p53, ras-responsive element, Pit 1, and PAX binding sites (supplemental Fig. S2).

**DISCUSSION**

Human *LOXL3* was previously identified in BLASTN searches of the GenBank™ databases (7, 22, 23). *LOXL3* is composed of 14 exons that encode a 753-amino acid long polypeptide with a calculated molecular mass of 83 kDa. An alternative splice variant of *LOXL3* with truncated SRCR domains was previously reported (22). The previously reported variant lacked the sequences corresponding to exons 5 and 8, but contained all the other exonic sequences (22), indicating that the variant arose by alternative splicing under the control of the same promoter. However, functional characterization of the previously identified variant has never been pursued.

Our newly identified variant *LOXL3-sv1* showed an exon-intron structure distinct from *LOXL3*, lacking exons 1, 2, 3, and 5. Furthermore, *LOXL3-sv1* mRNA contained additional sequences at both 5’ and 3’ termini; an 80-bp sequence corresponding to the intronic region proximal to the 5’-end of exon 4 in the 5’-UTR and a 561-bp sequence from the 3’-flanking intronic region of exon 14 in the 3’-UTR. The presence of these additional sequences in *LOXL3-sv1* cDNA indicates that two alternative promoters probably exist for expression of *LOXL3* and *LOXL3-sv1*. Using luciferase promoter assays, we showed that a strong promoter element exists in the 5’-flanking intronic region of exon 4 (*LOXL3-sv1* promoter). This is a distinct promoter element from the promoter region located in the 5’-proximal region of exon 1 (*LOXL3* promoter). The promoter region of *LOXL3* contains no typical canonical elements, TATA and CAAT boxes, in the proximity of the transcription initiation site but displays high CpG-rich sequences containing several potential AP-2 binding sites and single SP-1 site. The absence of TATA and CAAT boxes with a high CpG content in the promoter region, usually characteristic of euchromatic housekeeping genes (24), were also noticed in other LOX family genes (5, 6). In contrast, the *LOXL3-sv1* promoter contains a TATA box in the 5’-flanking region of exon 4 at positions +3196 to +3203 and, further, displays a different profile of potential transcription factor binding sites from the *LOXL3* promoter. This divergence in promoter structure suggests that differential regulation of those two alternative promoters may be responsible for different expression of *LOXL3* and *LOXL3-sv1*. In fact, the *LOXL3-sv1* promoter exhibited higher luciferase activity than the *LOXL3* promoter in skin fibroblasts, whereas the *LOXL3* promoter revealed much higher activity than the *LOXL3-sv1* promoter in HEK 293 cells, indicating that the *LOXL3* and *LOXL3-sv1* promoters are possibly subject to a cell-specific regulatory mechanism for the differential activation.

Processing of the LOX family proteins has been reported for LOX and LOXL (25, 26). The enzymatically active form of LOX with a molecular mass of 32 kDa results from extracellular pro-teolytic cleavage by procollagen C-protease, also known as bone morphogenic protein (BMP)-1, between residues Gly168 and Asp169 (25). BMP-1 was also shown to proteolytically cleave the LOXL precursor. The bovine LOXL protein was cleaved by BMP-1 at Gly130-Asp131 and Ser354-Asp355, resulting in two processed forms of LOXL (26). The BMP-1 cleavage site is exactly conserved in the human LOXL3 protein at Gly447-Asp449. Proteolytic cleavage at this potential BMP-1 recognition site is expected to result in a protein of a calculated molecular mass of 35 kDa. However, in our studies with the LOXL3-sv1 antibody that reacts to both LOXL3 and LOXL3-sv1, the 35 kDa protein was not detected in any tissues tested. Instead, a band with an apparent molecular mass of 40 kDa was detected in the colon tissue. Given the possibilities of post-translational modifications such as glycosylation, the 40-kDa protein may possibly correspond to the BMP1-processed form of LOXL3. Indeed, a couple of possible N-glycosylation sites are present within the predicted amino acid sequence of LOXL3 at residues 481–484 (Asn-Ile-Thr-Glu) and 625–628 (Asn-Gly-Thr-Lys), though further characterizations are required to
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LOXL3-sv1 showed the highest activity toward collagen type IV, a basement membrane-specific collagen, while LOXL3 presented the highest activity toward collagen type VIII, a network-forming collagen predominantly expressed in vascular endothelial cells and smooth muscle cells (32). These results suggest that LOXL3-sv1 may be involved in collagen type IV-mediated functions, such as cell adhesion, migration, differentiation, and growth, whereas LOXL3 possibly play a role in the maintenance of vessel wall integrity. Both LOXL3-sv1 and LOXL3 showed a relatively higher amine oxidase activity toward collagen types I and X than the other tested substrates, although to a lesser extent than toward collagen types IV and VIII. Collagen type I is the major structural protein in the extracellular matrix of skeletal tissues (33), and collagen type X is also known to be critical in endochondral bone formation (34). The higher amine oxidase activities of LOXL3 and LOXL3-sv1 toward these collagen types, thus, suggest a possible functional role for these two variants of amine oxidase in bone development or maintenance. Co-localization studies with these collagen types in human tissues will be helpful for further understanding of the functional roles of these amine oxidases in the biogenesis of extracellular matrices.

In summary, our findings indicate that alternative promoter activation possibly leads to expression of LOXL3-sv1 that functions as an amine oxidase with distinct tissue and substrate specificity from LOXL3. Recently, LOXL3 was reported to interact with Snail to down-regulate the expression of E-cadherin in carcinoma progression (35). However, the Snail-interacting parts of LOXL3 have yet to be determined. The absence of three N-terminal SRCR domains in LOXL3-sv1 suggests that LOXL3-sv1 possibly has a different interactive profile from LOXL3. Further elucidation of the interactive properties of LOXL3 and LOXL3-sv1 will provide significant insight into understanding the functional differences between these two variants of amine oxidase.

Acknowledgments—We thank Soo-Yeon Lee and Hye-Jin Lee for technical assistance.

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