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Amine Oxidase Activity of the Human Lysyl Oxidase-Like 3 (LOXL3) Protein

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Lysyl oxidase (LOX) catalyzes the lysine-derived cross-links of fibrillar collagens and elastin in the extracellular matrix. Recent molecular cloning has revealed existence of a LOX family consisting of LOX and four lysyl oxidase-like proteins (LOXL, LOXL2, LOXL3 and LOXL4). Pathological conditions associated with impaired LOX activity in several heritable and acquired disorders lead to severe structural and functional abnormalities of cardiovascular tissues, such as occlusion of coronary arteries and aneurysms, suggesting an essential role for the LOX family proteins in the maintenance of the cardiovascular system. However, the specific roles of the lysyl oxidase-like proteins in normal and pathological conditions of the cardiovascular tissues have not been established yet. Here, I report that LOXL3, a novel member of the LOX family, is predominantly expressed in the aorta, with an amine oxidase activity toward collagen and elastin, suggesting an essential role of LOXL3 in the development and maintenance of the aorta.

Key Words: Amine oxidase, Collagen, Cardiovascular diseases, Elastin, Extracellular matrix, Lysyl oxidase

INTRODUCTION

Lysyl oxidase (LOX) plays a critical role in morphogenesis and repair through the assembly of elastic fibers and collagen fibrils within the extracellular matrix of most tissues. LOX is known to oxidize peptidyl lysines in collagen and elastin to residues of α-aminoadipic δ-semialdehyde. The peptidyl aldehydes can then undergo spontaneous condensation with unreacted amino groups or neighboring aldehyde groups, thus forming the cross-links which convert elastin and collagen into insoluble fibers (Eyre et al., 1984; Smith-Mungo and Kagan, 1998).

Several distinct isoforms of LOX were identified in extracts of different tissues, each showing the same N-terminal residue and nearly identical peptide map (Kuivaniemi et al., 1984). These LOX isoforms presented similar but distinct amino acid composition, suggesting that these isoforms could be derived from distinct genes (Kuivaniemi et al., 1984). Recent molecular cloning has revealed that the LOX isoforms are encoded by the distinct LOX-like genes (LOXL, LOXL2, LOXL3 and LOXL4) (Asunction et al., 2001; Jourdan-Le Saux et al., 1999; Kim et al., 1995; Maki and Kivirikko, 2001; Saito et al., 1997). The derived amino acid sequences of all the lysyl oxidase-like genes show significant sequence homology to LOX within the C-terminus containing a copper binding motif, residues for lysyl tyrosylquinone (LTQ), and a cytokine receptor-like (CRL) domain. In addition to the conserved C-terminal region, LOXL2, LOXL3, and LOXL4 contain four repeated copies of a scavenger receptor cysteine rich (SRCR) domain at the N-terminus (Asunction et al., 2001; Jourdan-Le Saux et al., 1999; Maki and Kivirikko, 2001), implying an additional function of this subgroup of the LOX family.

The different expression patterns of the LOX family proteins in human tissues indicated a unique function for each of these potential amine oxidases (Asunction et al., 2001; Jourdan-Le Saux et al., 1999; Kim et al., 1995; Maki and Kivirikko, 2001; Saito et al., 1997). However, it has not been established what specific roles these LOX-like proteins play in extracellular matrices. In this study, we report that LOXL3 functions as an active amine oxidase toward physiological substrates of the LOX family, such as collagen and elastin. Furthermore, LOXL3 is predominantly expressed

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in the adult aorta, possibly indicating a unique function for LOXL3 in the development and maintenance of the aorta.

MATERIALS AND METHODS

1. Buffers

Buffer A contains 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF and 1 mg/ml of lysozyme. Buffer B contains 2 M urea and 10 mM K_2HPO_4, pH 8.2. Buffer C contains 8 M Urea, 10 mM K_2HPO_4, pH 8.2 and 3 mM β-mercaptoethanol. Buffer D contains 10 mM K_2HPO_4, pH 7.0. Buffer E contains 10 mM K_2HPO_4, pH 9.6, 200 μM $CuCl_2$ and 2% sodium N-lauroylsarcosinate. Buffer F contains 10 mM K_2HPO_4, pH 9.6 and 5 μM $CuCl_2$.

2. Construction of the LOXL3 expression plasmid

The LOXL3 cDNAs were PCR-amplified from human placental total RNA (BD Biosciences) using the *Pfu* polymerase (Promega) according to the manufacturer's suggestions. The recombinant protein LOXL3 was designed to start the open-reading frame at Leu^{233} with an additional Met-Ala-Ser sequence at the N-terminus and a hexa-histidine tag at the C-terminus. The sequences of oligonucleotide primers used for cloning of the LOXL3 cDNA are 5'-atagctagcctagccccaacggcagcaacactccttta-3' for the forward primer and 5'-gcgaagcttgataatctggttgctggtctg-3' for the reverse primer. The forward and reverse primers contained a *Nhe*I or *Hind*III restriction site, respectively, for convenient subcloning. Thermocycling consisted of 30 cycles at 94℃ for 60 s, 60℃ for 60 s and 72℃ for 60 s with a predenaturation at 94℃ for 2 min and a final extension at 72℃ for 5 min. The PCR-amplified DNA fragment was gel-purified and then ligated into pET21b (Novagen) at the *Nhe*I and *Hind*III restriction sites in frame with the C-terminal hexahistidine tag. The resulting expression construct pET21b-LOXL3 was 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.

3. Expression and purification of the LOXL3 protein

All the purification and refolding procedures were carried out at 4℃ unless otherwise indicated. The pET25b-derived expression construct was transformed in *E. coli* BL21 (DE3) (Novagen). The transformants were grown in 1 L of LB medium containing 100 μg/ml of ampicillin at 37℃ until the OD_{600} of culture reached 0.6. For induction, 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added. After 4 h of induction, the transformants were harvested by centrifugation at 7,000g for 20 min and resuspended in 60 ml of buffer A. Triton X-100 (1%; w/v) and DNase (0.1 mg/ml) were sequentially added to the lysates at intervals of 30 min during inverted mixing, and then sonication was repeated twice at 70% efficiency with a sonicator (Sonics & Materials). Inclusion body fractions were collected by centrifugation at 8,000g for 20 min and washed in 60 ml of buffer B. The washed inclusion bodies were solubilized in 60 ml of buffer C, incubated overnight with constant mixing, and filtered through a 0.45 μm syringe filter (Sartorius). The solubilized inclusion bodies were purified using the Ni-NTA agarose resins (Qiagen) according to the manufacturer's recommendations. The purity of the recombinant protein was analyzed by SDS-PAGE.

4. Refolding of the LOX and LOXL proteins and analysis of residual chemicals

In an attempt to refold the proteins denatured by urea, the protein samples were subjected to consecutive dialyses. First, the purified proteins were diluted to 100 μg/ml in buffer D. The diluted proteins were dialyzed overnight first in buffer E containing 2% of sodium N-lauroylsarcosinate and then against buffer F. The proteins were further dialyzed twice against buffer D overnight. The concentration of dialyzed protein samples was determined by the BCA method (Smith et al., 1985). After dialysis, the protein solutions were aliquoted and lyophilized using a Freeze dryer (Labconco). Trehalose was added to the protein solutions at a final concentration of 10 mM before lyophilization. Samples were frozen at -40℃, dried for 24 h, capped within vacuum and then stored at -20℃. Residual chemicals in the purified proteins, such as sodium N-lauroylsarcosinate, imidazol, β-mercaptoethanol and urea, were analyzed at 210 nm by HPLC equipped with a μ-Bondapack C18 reverse phase column (Waters Associates; 300×3.9 mm). The column was pre-equilibrated with the buffer of 0.2 mM K_2HPO_4, pH 7.0, and the samples were eluted in the solvent of 100% acetonitrile with a linear gradient of 0~40% over 20 min at a flow rate of 1 ml/min.

5. In vitro amine oxidase assays

The amine oxidase activity of the LOXL3 recombinant protein was measured using a peroxidase-coupled fluorometric assay with the Amplex red hydrogen peroxide assay kit (Molecular Probes) as previously described (Palamakumbura and Trackman, 2002). Each reaction contained 10 μg of the LOXL3 protein and 1 mM of a substrate. Elastin from bovine neck ligament, collagen type I from calf skin, collagen type II from the chicken sternal cartilage, collagen type III from calf skin, and collagen types IV from human placenta was used as substrates, all purchased from SIGMA. Parallel assays were performed in the absence or presence of 1 mM BAPN. Fluorescence was measured using a fluorescence spectrophotometer (Molecular Devices) with excitation and emission wavelengths of 571 nm and 585 nm. All assays were repeated in quadruplicate, and the results were expressed as the fluorescence increase over that of BAPN controls.

6. Northern blot analysis

A human cardiovascular tissue membrane (Clontech) was prehybridized for 4 h at 42℃ in 10 ml of a solution containing 50% formamide, 5X SSPE, 10X Denhardt's solution, 2% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization, using a P^{32}-labeled LOXL3 cDNA probe prepared by Prime It (Stratagene), was carried out in 6 ml of the same solution used for prehybridization at 42℃ for overnight. The filters were then washed twice in 2X SSC, 0.1% SDS at room temperature and twice in 0.1X SSC, 0.1% SDS at 42℃. The washed filters were then exposed to Kodak XAR films at -70℃. GAPDH was used as an internal control.

RESULTS

1. Northern blot analysis of LOXL3 in the human cardiovascular tissues

Previously, the 3.1 kb human LOXL3 mRNA was predominantly detected only in the heart and placenta (Maki and Kivirikko, 2001). In an attempt to further access the expression of LOXL3 in the human cardiovascular tissues, we performed Northern blot analysis of LOXL3 with the human cardiovascular tissues (Fig. 1). The LOXL3 expression was almost exclusively restricted to the aorta. In contrast, the LOXL3 mRNA was barely detected in the fetal heart, the adult total heart, the apex of heart, the left and right atriums, and the left and right ventricles (Fig. 1).

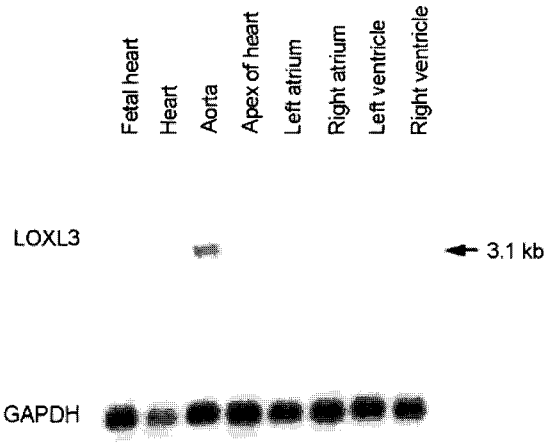

Fig. 1. Northern blot analysis of LOXL3 in cardiovascular tissues. A human cardiovascular tissue blot containing 2 μg of polyA$^+$ RNA samples was incubated with a radio-labeled LOXL3 cDNA probe. GAPDH was used as an internal control. The size of the LOXL3 mRNA is indicated with an arrow.

2. Expression and purification of the recombinant LOXL3 protein

In an effort to express and purify an enzymatically active form of LOXL3, an expression plasmid of LOXL3 was constructed using an *E. coli* expression plasmid pRT21b (Fig. 2A). Upon induction with 1 mM IPTG at 37℃, the hexa-histidine tagged recombinant protein was expressed at a high level from the expression construct. The apparent size (60 kDa) of the expressed recombinant protein was in good agreement with the deduced molecular mass (Fig. 2B) Fractionation of the cell lysates into different cellular compartments, such as cytoplasmic extracts, periplasmic extracts and inclusion body fractions, revealed that the recombinant LOXL3 protein was expressed within the inclusion bodies. The insoluble recombinant protein was denatured by urea during purification and was, subsequently, refolded by stepwise dialysis in the presence of N-lauroylsarcosinate and Cu^{2+}. The recombinant protein was determined to be more than 95% pure on SDS-PAGE gel (Fig. 2C).

3. Amine oxidase activity of LOXL3

The refolded recombinant LOXL3 protein was assessed for amine oxidase activity toward collagen and elastin using

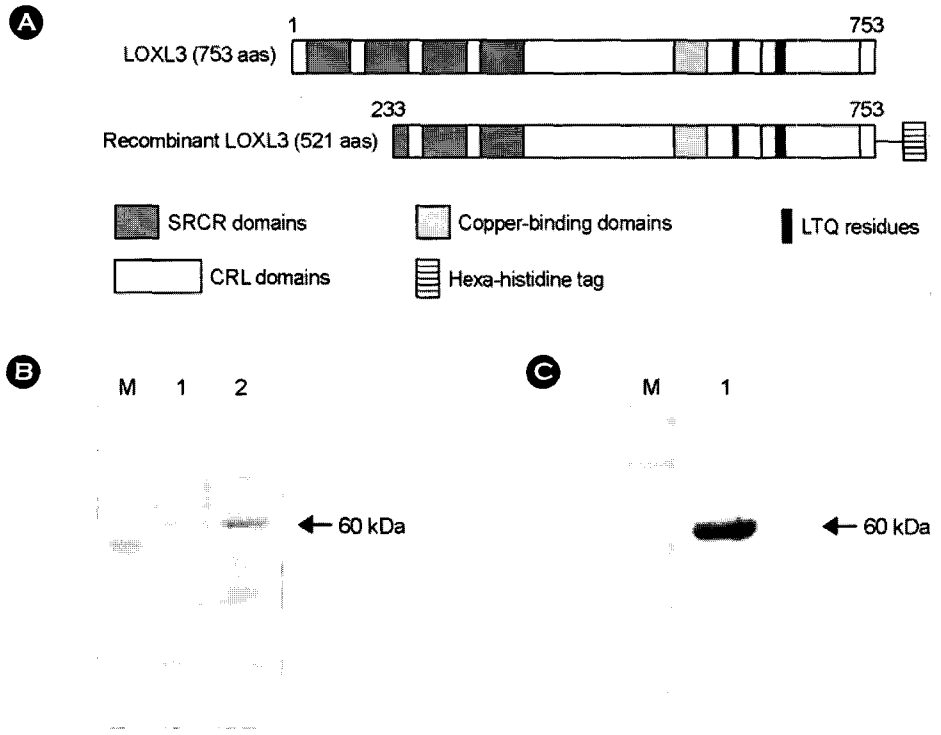

Fig. 2. Expression and Purification of LOXL3. **(A)** Schematic diagrams of LOXL3 and the recombinant LOXL3 protein. The recombinant LOXL3 protein starts at Leu^{233} and contains an addi- tional Met-Ala-Ser sequence at the N-terminus and a hexa-histidine tag at the C-terminus. **(B)** Expression of LOXL3 in *E. coli*. Total proteins were extracted from 1 ml of bacterial transformants either uninduced (lane 1) or induced by 1 mM IPTG (lane 2). Lane M contains molecular mass standards. The arrow indicates the expected molecular mass of the recombinant LOXL3 protein. **(C)** Purification of the recombinant LOXL3 protein. Lane 1 contains approximately 5 μg of the purified recombinant LOXL3 protein. Lane M contains molecular mass standards.

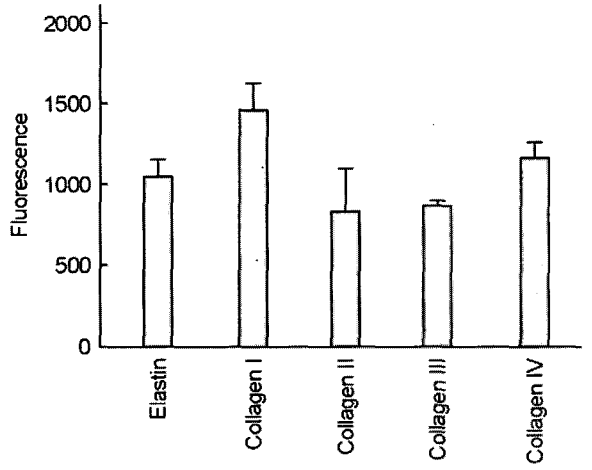

Fig. 3. Amine oxidase activity of the recombinant LOXL3 protein toward elastin and collagen. The amine oxidase assays were repeated in quadruplicate. Parallel assays were performed in the absence or presence of 1 mM BAPN. For each activity, shown is the measured increase in fluorescence over that of BAPN controls. The standard deviations are also indicated on the graph.

a peroxidase-coupled fluorometric assay. Physiological substrates of the LOX family proteins, such as elastin and collagen types I, II, III, and IV, were tested as substrates for the amine oxidase assays of the recombinant LOXL3 protein. The LOXL3 protein showed significant levels of amine oxidase activity toward all the tested substrates but presented higher substrate specificities toward collagen types I and IV than toward the other substrates tested (Fig. 3). All the amine oxidase activities were significantly inhibited by 100 molar excess of BAPN, an irreversible specific inhibitor of LOX, indicating that the fluorometric activities in the assays were originated from the recombinant LOXL3 protein.

DISCUSSION

Human LOXL3 was previously identified in BLASTN searches of the GenBank databases (Huang et al., 2001; Jourdan-Le Saux et al, 2001; Maki and Kivirikko, 2001). LOXL3 is composed of 14 exons that encode a 753 amino acid-long polypeptide with a calculated molecular mass of 83 kDa. However, functional characterization including

the enzymatic activity of LOXL3 has never been pursued. LOXL3 contain the characteristic C-terminal domains of the LOX family, such as the copper-binding domain and the CRL domain. The copper-binding domain (WXWH-XCHXHYH; where X is any amino acid) is distinguished by the presence of four histidine residues, which has been postulated to form an octahedral coordination complex with copper ion (Krebs and Krawetz, 1993). The CRL domain ($C\text{-}X_9\text{-}C\text{-}X\text{-}W\text{-}X_{34}\text{-}C\text{-}X_{13}\text{-}C$; where X_n is a defined number of any amino acids) of the LOX family was also found in the extracellular domains of a number of different receptors for cytokines, prolactin, and growth hormones (Miyajima et al., 1992). The four cysteine residues sparsely located in this domain were suggested to form two disulfide bonds, which are believed to be important for ligand binding in the growth factor and cytokine receptor superfamily (Miyajima et al., 1992).

The strict conservation of the C-terminal domains indicates that LOXL3, similarly to LOX, also functions as an amine oxidase. In this report, using an *E. coli* over-expression system and stepwise dialysis for refolding of the over-expressed recombinant protein, we successfully show that the human LOXL3 protein functions as an amine oxidase toward physiological substrates of LOX such as collagen and elastin proteins.

In the cardiovascular system, LOX plays an important role both in morphogenesis and repair. Multiple cardiovascular abnormalities arise in acquired and inherited disorders with impaired LOX activities. Fibrous tissue accumulation is an integral feature of the adverse structural remodeling of cardiac tissue seen in many cardiovascular diseases. In arterial hypertension, for example, accumulation of fibrillar collagen type I and III occurs throughout the walls and interventricular septum of the heart (Pardo-Mindan and Panizo, 1993; Schwartzkopff et al., 1993). This increase in collagen content raises myocardial stiffness and promotes abnormalities of cardiac function and electrical activity (Dixon et al., 1996; Mclenachan and Dargie, 1990). The LOX protein levels have been shown to correlate with the degree of the disease in hypertensive rats (Bergomi et al., 1997; Capdeville et al., 1989; Sheridan et al., 1979). In early atherosclerotic lesions of the aorta, total collagen and elastin contents are known to be significantly elevated in the affected intima and media (Ross, 1993). LOX enzyme levels have been shown to increase after induction of myocardial infarction in rabbits by coronary ligation (Kagan et al., 1981) In our Northern blot analysis, LOXL3 was predominantly expressed in the adult aorta. Further, in the amine oxidase assays, the LOXL3 protein showed a BAPN-inhibitable amine oxidase activity toward all the tested substrates including collagen types I, II, III, and IV and elastin, suggesting a significant role of LOXL3 in the morphogenesis and repair of the aorta.

In summary, these findings suggest that LOXL3 possibly possesses a potential role as an aorta-specific amine oxidase. Further characterizations such as colocalization studies of LOXL3 with various types of collagens and elucidations of the interactive profiles of LOXL3 will be significantly helpful in developing therapeutic treatments for the cardiovascular diseases associated with impaired LOXL3 activity.

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