Molecular Cloning and Biological Activity of a Novel Lysyl Oxidase-related Gene Expressed in Cartilage*

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We cloned a cDNA encoding a novel lysyl oxidase-related protein, named LOXC, by suppression subtractive hybridization between differentiated and calcified ATDC5 cells, a clonal mouse chondrogenic EC cell line. The deduced amino acid sequence of mouse LOXC consists of 757 amino acids and shows 50% identity with that of mouse lysyl oxidase. Northern blot analysis showed a distinct hybridization band of 5.4 kilobases, and Western blot analysis showed an immunoreactive band at 82 kilodaltons. Expression of LOXC mRNA was detected in osteoblastic MC3T3-E1 cells and embryonic fibroblast C3H10T1/2 cells, whereas none of NIH3T3 fibroblasts and myoblastic C2C12 cells expressed LOX mRNA in vitro. Moreover, the LOXC mRNA and protein levels dramatically increased throughout a process of chondrogenic differentiation in ATDC5 cells. In vivo, LOX gene expression was localized in hypertrophic and calcified chondrocytes of growth plates in adult mice. The conditioned media of COS-7 cells transfected with the full-length LOXC cDNA showed the lysyl oxidase activity in both type I and type II collagens derived from chick embryos, and these activities of LOXC were inhibited by β-aminopropionitrile, a specific inhibitor of lysyl oxidase. Our data indicate that LOXC is expressed in cartilage in vivo and modulates the formation of a collagenous extracellular matrix.

Endochondral bone formation is a multistep programmed event in skeletal development. Undifferentiated mesenchymal chondroprogenitor cells differentiate into chondrocytes through a cellular condensation process. Such chondrocytes surround themselves with an abundant layer of extracellular matrix, including type II, IX, and XI collagens, that is characteristic of cartilage (1, 2). These cells go through sequential processes of proliferation and maturation and then change their genetic program to be converted into hypertrophic and calcified chondrocytes, expressing type X collagen. These events are under the regulatory control of a variety of growth and differentiation modulating factors, including bone morphogenetic proteins (3, 4), fibroblast growth factors (5, 6), parathyroid hormone-related peptide (7–9), and Indian hedgehog (10). It is also clear that the components of extracellular matrix in cartilage play important roles in modulating and maintaining the phenotype of chondrocytes. During a process of hypertrophic conversion and calcification of chondrocytes, mineralization of extracellular matrix occurs before these chondrocytes are replaced by bone tissues. However, the molecular mechanisms underlying these sequential events remain largely unknown.

We previously reported that the clonal mouse cell line, ATDC5, enables the monitoring of the multistep chondrogenic differentiation in a single culture (11–14). When cultured in the presence of insulin, ATDC5 cells form cartilaginous nodules through cellular condensation. When the formation of cartilage nodules is completed, the cells are then converted to type X collagen-expressing hypertrophic chondrocytes, following the process of mineralization. By taking advantage of the fact that these chondroprogenitor-like cells undergo sequential differentiation in a synchronous manner, we compared mRNAs expressed in hypertrophic and calcified ATDC5 cells, expressing type X collagen, with those in differentiated ATDC5 cells, before expressing type X collagen, by suppression subtractive hybridization, and we isolated a novel cDNA clone encoding a lysyl oxidase-related protein expressed in cartilage as well as ATDC5 cells.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—ATDC5 cells were plated in 6-multilwell plates at an initial cell density of 6 × 10⁴ cells/well and were cultured as described previously (11–15). Briefly, cells were cultured for the initial 21-day period in Dulbecco’s modified Eagle’s medium/Ham’s F-12 hybrid medium (ICN Pharmaceuticals, Inc., Costa Mesa, CA) containing 5% fetal bovine serum (Life Technologies, Inc.), 10 μg human transferrin (Roche Molecular Biochemicals), 30 μg sodium selenite (Sigma), and 10 μg bovine insulin (Wako Pure Chemical, Osaka, Japan) at 37 °C in a humidified 5% CO₂, 95% air atmosphere. On day 21, the culture medium was switched to α-minimum essential medium (ICN Pharmaceuticals, Inc.) containing 5% fetal bovine serum, 10 μg human transferrin, 30 μg sodium selenite, and 10 μg bovine insulin, and the CO₂ concentration was shifted to 3% for facilitating cellular hypertrophy and mineralization in culture. Clonal mouse embryonic fibroblast C3H10T1/2 cells, clonal mouse osteogenic MC3T3-E1 cells, clonal mouse fibroblast NIH3T3 cells, and clonal mouse myoblastic C2C12 cells (RIKEN Cell Bank, Tsukuba, Japan) were plated in 6-multwell plates at an initial cell density of 6 × 10⁴ cells/well and cultured as described previously (16).

RNA Extraction and Suppression Subtractive Hybridization—Poly(A)⁺ RNA was isolated from differentiated (day 21) and calcified (day 42) ATDC5 cells by a single-step method as described previously (17) and analyzed by suppression subtractive hybridization according to the manufacturer’s instructions (PCR-Select cDNA Subtractions Kit, CLONTECH Laboratories, Inc., Palo Alto, CA). The cDNA fragment of...
600-base pairs (bp) expressed at a high level in calcified ATDC5 cells was identified and subcloned into pCR 2.1 vector (Invitrogen Co., San Diego, CA).

cDNA Library Construction and Isolation of Mouse LOXC cDNA—
The oligo(dT)-primed cDNA library from poly(A) RNA of calcified ATDC5 cells was constructed in ZAP Express vector (Stratagene, La Jolla, CA), and 1 × 10⁶ plaques were screened with the cDNA fragment as a probe (12, 16). Plaques were transferred to the membranes (137-mm nylon membrane, PerkinElmer Life Sciences), and the 600-bp fragment was 32P-labeled (BcaBEST labeling kit, Takara, Otsu, Japan), and hybridization was performed as described previously (12). The membranes were washed to a final stringency of 0.1× SSPE (3 M NaCl, 197 mM NaH₂PO₄, 25 mM EDTA), and 0.1% SDS at 55 °C. The nucleotide sequence was determined with ALFred DNA Sequencer (Amersham Pharmacia Biotech).

Northern Analysis—ATDC5 cells, C3H10T1/2 cells, MC3T3-E1 cells, NIH3T3 cells, and C2C12 cells were plated in 6-multiwell plastic plates and cultured as described above. Total RNA from various cell lines cultured in vitro as well as poly(A) RNA from rib cartilage of 3-week-old ICR mice were isolated and analyzed by Northern hybridization as described previously (12, 17). Briefly, total RNA (20 μg) and poly(A) RNA (2 μg) were denatured, separated by 1% agarose gel electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell). A 1.3-kilobase pair (kb) cDNA fragment of LOXC, a 0.55-kb cDNA fragment of mouse type II collagen, and a 0.65-kb cDNA fragment of mouse type X collagen were used for hybridization as probes. In analysis of tissue distribution in adult mice, a labeled cDNA was hybridized to a mouse multiple tissue Northern blot (CLONTECH Laboratories, Inc.). After hybridization, the membranes were exposed to X-Omat films (Eastman Kodak Co.) at 280 °C with Cronex Lightning Plus intensifying screen (PerkinElmer Life Sciences).

In Situ Hybridization—Tibiae of male neonate C57BL/6J mice were collected and fixed in 4% paraformaldehyde in 10 mM phosphate-buffered saline, pH 7.4, overnight at 4 °C. Tibiae were decalcified for 4 days in 10% EDTA. They were dehydrated in a graded series of ethanol and embedded in paraffin. Sections (6 μm thick) were then processed for in situ hybridization as described previously (18). Subclones of the 1.3-kb mouse LOXC cDNA, the 0.4-kb mouse type II collagen cDNA, and the 0.55-kb mouse type X collagen cDNA into pBSII-KS(+) (Stratagene) were linearized with appropriate restriction enzymes to transcribe either sense or antisense 35S-labeled riboprobes. After hybridization, the slides were washed under conditions of high stringency, and the dried slides were mounted in a graded series of ethanol and embedded in paraffin. Sections (6 μm thick) were then processed for in situ hybridization as described previously (18). In addition, the sections pretreated with RNase before in situ hybridization with the riboprobes showed no autorgraphic signals, indicating that the hybridization signals were dependent on the presence of RNA (data not shown).

In Vitro Transcription/Translation—A coupled transcription/translation reaction was performed using the rabbit reticulocyte lysate sys-

The abbreviations used are: bp, base pair; kb, kilobase pair; βAPN, β-aminopropionitrile.

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Fig. 1. Structure of mouse LOXC cDNA. Nucleotide sequence of mouse LOXC and the deduced amino acid sequence. The GenBank™ accession number for mouse LOXC is AF338340.

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tem (TnT-coupled Transcription/Translation Systems, Promega Co., Madison, WI) in the presence of \[^{35}S\]methionine (Amersham Pharmacia Biotech, catalog number AG1094) according to the manufacturer's instructions. The 5.4-kb mouse LOXC cDNA cloned into pcDNA3.1 vector was used as a template (pCMV/mLOXC). The translation product was electrophoresed on a 10–20% polyacrylamide gel and

![FIG. 2. Comparison of the amino acid sequences of LOXC, lysyl oxidase-related protein 2 (Lor2), and lysyl oxidase (LO). Identical residues are shown in gray. Conserved cysteine residues are shown in black. The putative cleavage site is indicated by an arrow. The four scavenger receptor cysteine-rich domains are overlined and numbered in parentheses. The putative copper-binding site and the GHK site are boxed. The Lys and Tyr residues that form lysyl tyrosylquinone by cross-linking are indicated by the arrowheads. The amino acid sequences of mouse lysyl oxidase and mouse lysyl oxidase-related protein 2 are deduced from the nucleotide sequences of the GenBank accession numbers, NM010728 and AF053368, respectively.](image)

![FIG. 3. Translation of LOXC gene in vitro. A coupled transcription/translation reaction (TnT Reticulocytes, Promega) was performed using mouse LOXC cDNA cloned into pcDNA3.1 vectors as a template. The translation products were separated by electrophoresis on a gradient polyacrylamide gel (10–20%) and detected by autoradiography. Three independent experiments were performed and gave similar results.](image)

![FIG. 4. Expression of LOXC protein. pCMV/mLOXC, or pcDNA3.1 vector as a control, was transiently transfected into COS-7 cells, and LOXC protein was detected using anti-LOXC antibody. Original magnification, × 100. Three independent experiments were performed and gave similar results.](image)
Northern blot hybridization using 1.3-kb LOXC cDNA. (undifferentiated and calcified), NIH3T3, or C2C12, was analyzed by per lane of total RNA extracted from each cells, C3H10T1/2, MC3T3-E1 0.55-kb type II collagen cDNA, and 0.65-kb type X collagen cDNA (indicated and analyzed by Northern blot using 1.3-kb LOXC cDNA, of total RNA and the conditioned media were prepared at the time point experiments were performed and gave similar results.

Western Blot Analysis—

DNA Transfection of COS-7 Cells—COS-7 cells were cultured in Dulbecco's modified Eagle's medium (ICN Pharmaceuticals, Inc.) containing 10% fetal bovine serum. For the assay of lysyl oxidase activity, COS-7 cells were transiently transfected with pCMV/mLOXC or pcDNA3.1 by using DEAE-dextran (Sigma), and the conditioned media were prepared 7 days after transfection. For Western blot analysis, COS-7 cells were transfected with pCMV/mLOXC or pcDNA3.1 by using FuGene6 (Roche Molecular Biochemicals) according to the manufacturer's instructions, and total cellular proteins were prepared 2 days after transfection.

Western Blot Analysis—Total cellular proteins were prepared from ATDC5 cells and COS-7 cells transfected with pCMV/mLOXC or pcDNA3.1 as described previously (17). Forty micrograms of protein were separated by a 10–20% polyacrylamide gel and transferred to nitrocellulose filters. The filters were blocked in 3% gelatin in Tris-buffered saline containing 0.1% Tween 20 and then incubated with the anti-LOXC antibody. Prestained rainbow marker (Bio-Rad) was loaded in the adjacent lane to estimate a molecular size.

Fig. 5. Expression of LOXC mRNA in various cell lines. A, 20 μg per lane of total RNA extracted from each cells, C3H10T1/2, MC3T3-E1 (undifferentiated and calcified), NIH3T3, or C2C12, was analyzed by Northern blot hybridization using 1.3-kb LOXC cDNA. B and C, ATDC5 cells were cultured described under "Experimental Procedures." 20 μg of total RNA and the conditioned media were prepared at the time point indicated and analyzed by Northern blot using 1.3-kb LOXC cDNA, 0.55-kb type II collagen cDNA, and 0.65-kb type X collagen cDNA (B) and by Western blot using anti-LOXC antibody (C). Three independent experiments were performed and gave similar results.

Fig. 6. Expression of LOXC mRNA in various adult mouse tissues. A, multiple tissue blot containing 2 μg of poly(A)+ RNA from various mouse tissues (CLONTECH) was hybridized with the LOXC cDNA (top) and a β-actin probe (bottom). The mRNA in each lane was isolated from 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; and 8, testis. A DNA fragment of β-actin was also hybridized to the same blot as a control. B, 2 μg of poly(A)+ RNA from rib cartilage was hybridized with the LOXC cDNA (top) and a β-actin probe (bottom). Three independent experiments were performed and gave similar results.

Assay of Lysyl Oxidase Activity—Preparation of collagen substrates and assay of lysyl oxidase activity were performed as described previously (20). In brief, 20 pairs of calvariae or growth plates of proximal tibiae in 17-day-old chick embryos were suspended in 6 ml of Eagle's minimal essential medium without lysine (Life Technologies, Inc.). The tibiae in 17-day-old chick embryos were suspended in 6 ml of Eagle's minimal essential medium without lysine (Life Technologies, Inc.). The substrate solution containing 3H-labeled type I or type II collagen substrates and assay of lysyl oxidase activity were performed as described previously (20). In brief, 20 pairs of calvariae or growth plates of proximal tibiae in 17-day-old chick embryos were suspended in 6 ml of Eagle's minimal essential medium without lysine (Life Technologies, Inc.). The substrate solution containing 3H-labeled type I or type II collagen substrates was added to the conditioned media. The substrate solution containing 3H-labeled type I or type II collagen substrates was added to the conditioned media. The substrate solution containing 3H-labeled type I or type II collagen substrates was added to the conditioned media. The substrate solution containing 3H-labeled type I or type II collagen substrates was added to the conditioned media. The substrate solution containing 3H-labeled type I or type II collagen substrates was added to the conditioned media. Then, the mixture was incubated at 37 °C for 24 h. The calvariae or the growth plates were then homogenized in 0.05 M Tris-HCl buffer, pH 7.4, containing 1 mM NaCl. The homogenates were stirred for 90 min at 4 °C and then centrifuged at 20,000 × g for 20 min. Labeled collagens in the supernatant fraction were precipitated by salting with 20% NaCl and collected by centrifugation at 30,000 × g for 20 min. The precipitates were suspended in a minimum volume of 0.05 M Tris acetate buffer, pH 7.7, containing 0.15 M NaCl and dialyzed against the same buffer for 24 h.

The substrate solution containing 3H-labeled type I or type II collagen equivalent to 2.5 × 105 cpm was added to each assay tube. To this solution was added 0.9 ml of the conditioned media of COS-7 cells described above. The reaction mixture was incubated at 37 °C for 2 h, and the reaction was stopped by freezing the mixture at −20 °C. Trinitiated water formed was collected by vacuum distillation according to the method of Pinnell and Martin (21), and 0.8-ml portions of the distillate were counted in a liquid scintillation counter. Assays were also carried out in the presence of βAPN (50 μg/ml), a specific inhibitor of lysyl oxidase, after preincubation with 3H-labeled collagen substrates for 1 h prior to the addition of the conditioned media.
CDNA library generated from calcified ATDC5 cells. Twenty was then used as a probe to screen at high stringency a mouse sequence of a novel gene named \textit{LOXC}. A 600-bp cDNA fragment corresponding to the 3'-end of the sequence contains a poly(A) stretch, preceded by putative polyadenylation signals (AATAAA). The 25 amino acids, starting from the first ATG initiation codon, possessed features characteristic of signal peptide sequences. The data suggest that the LOXC cDNA is likely to encode an extracellular protein. Cleavage of the signal peptide would yield a protein of 732 amino acids residues, having a calculated molecular mass of 84,705 Da and an isoelectric point of 7.8. The 3'-end of the sequence contains a poly(A) stretch, preceded by putative polyadenylation signals (AATAAA). The 25 amino acids, starting from the first ATG initiation codon, possessed features characteristic of signal peptide sequences. The data suggest that the LOXC cDNA is likely to encode an extracellular protein. Cleavage of the signal peptide would yield a protein of 732 amino acids residues, having a calculated molecular mass of 84,705 Da and an isoelectric point of 7.8.

Expression of LOXC mRNA in the Adult Mice Tissues and Growth Plates of Neonate Mice—Northern analysis showed that among the various adult mice tissues, LOXC mRNA was expressed in cartilage and not in heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (Fig. 6, A and B). By \textit{in situ} hybridization, LOXC mRNA was localized in hypertrophic and calcified chondrocytes of growth plates in neonate mice. LOXC mRNA level increased in parallel with the induction of type II collagen mRNA level in the culture and peaked on day 7 (Fig. 5B). The level declined until day 14 and then increased until cells became calcified on day 42. As shown in Fig. 5C, LOXC protein was not detected in undifferentiated ATDC5 by Western blot analysis, and the protein level in the culture increased during a process of chondrogenic differentiation in these cells.

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Lysyl Oxidase Enzyme Activity of LOXC—The deduced amino acid sequence of LOXC showed homology with that of lysyl oxidase, suggesting that LOXC may possess the lysyl oxidase enzyme activity. We assessed such activity by a tritium release assay procedure using type I and type II collagen substrates prepared from chick embryos. LOXC protein was produced in the conditioned media of COS-7 cells transfected with LOXC cDNA and was detected by Western blot analysis. Significant activities were detected in the conditioned media of
COS-7 cells transfected with LOXC cDNA but not with the control vector (Fig. 8). Moreover, these enzyme activities were inhibited by βAPN, a mechanism-based, irreversible inhibitor of lysyl oxidase.

**DISCUSSION**

We have isolated by suppression subtractive hybridization a novel gene, LOXC, encoding a protein of 757 amino acids, which is expressed in cartilage in vivo. The amino acid sequence of mouse LOXC showed 50% identity with that of a mouse lysyl oxidase. Lysyl oxidase is an extracellular, copper-dependent enzyme that initiates covalent cross-linking between and within the molecular units of collagens by catalyzing the oxidative deamination of peptidyl lysine in these proteins to peptidyl α-aminoadipic-δ-semialdehyde (22). Lysyl oxidase is synthesized as a preproprotein, secreted as a 50-kDa proenzyme, and then proteolytically cleaved to the 32-kDa catalytically active, mature enzyme. There are three lines of evidence for the notion that LOXC is an extracellular protein secreted as an 82-kDa active, mature enzyme. First, LOXC protein has a putative signal peptide at its N-terminal (Fig. 2). Second, amino acid sequence alignment revealed that LOXC contains four repeats of the scavenger receptor cysteine-rich domains, found in diverse secreted and cell membrane-associated proteins (23). Finally, LOXC protein was recognized as a specific band of 82-kDa in the conditioned media of COS-7 cells transfected with LOXC cDNA on Western blotting with anti-LOXC antibody.

Lysyl oxidase contains one tightly bound copper (II) cofactor (22). Two sequence motifs important for the binding to copper have been determined, which are highly conserved among different lysyl oxidase proteins, WXXH and CHXHXXH is involved in the copper binding coordination and includes the four histidines that supply the nitrogen ligands, and GHK, another putative collagen-related copper affinity site, is also conserved in LOXC (Fig. 2). Lysyl oxidase also contains a lysine tyrosylquinone as a carbonyl cofactor (24). The Tyr and Lys residues in the C-terminal of lysyl oxidase participate together in the formation of this cofactor, and these Lys (amino acid 639) and Tyr (amino acid 675) residues are conserved in LOXC (Fig. 2). These data raise the possibility that LOXC possesses the lysyl oxidase enzyme activity and is involved in cross-linking of extracellular matrix. Indeed, the conditioned media of COS-7 cells transfected with LOXC cDNA exhibited significant lysyl oxidase enzyme activity, using chick 3H-labeled type I and type II collagen substrates. Moreover, these enzyme activities were inhibited by βAPN.

Endochondral bone formation includes a cascade of cellular events such as cellular condensation of chondroprogenitor cells, proliferation, maturation, hypertrophic conversion, calcification of chondrocytes, and the cartilage replacement by bone. During these processes, collagenous components of the extracellular matrix are transitionally changed from type II collagen to type X collagen. Furthermore, condensed chondroprogenitors and the chondro-osseous mineralizing border exhibit the expression of type I collagen. Although the expression pattern of LOXC protein in the growth plate remains to be elucidated, our in vitro and in vivo data that LOXC is expressed in condensed chondroprogenitor cells and hypertrophic and calcified chondrocytes and possesses lysyl oxidase enzyme activity provide evidence for the hypothesis that LOXC may regulate the endochondral bone formation by modulating collagenous extracellular matrix.

In this study, we identified LOXC gene by suppression subtractive hybridization to screen genes highly expressed in hypertrophic and calcified ATDC5 cells. Our observations that LOXC is expressed in hypertrophic and calcified chondrocytes in vitro and certain cell lines in vitro and that LOXC has lysyl oxidase enzyme activity raise the possibility that LOXC may play a critical role in endochondral bone formation.

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