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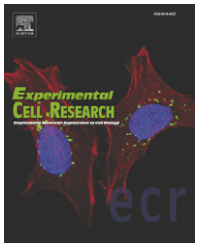

Research Article

The transcription factor Lc-Maf participates in *Col27a1* regulation during chondrocyte maturation

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ABSTRACT

The transcription factor Lc-Maf, which is a splice variant of c-Maf, is expressed in cartilage undergoing endochondral ossification and participates in the regulation of type II collagen through a cartilage-specific *Col2a1* enhancer element. Type XXVII and type XI collagens are also expressed in cartilage during endochondral ossification, and so enhancer/reporter assays were used to determine whether Lc-Maf could regulate cartilage-specific enhancers from the *Col27a1* and *Col11a2* genes. The *Col27a1* enhancer was upregulated over 4-fold by Lc-Maf, while the *Col11a2* enhancer was downregulated slightly. To confirm the results of these reporter assays, rat chondrosarcoma (RCS) cells were transiently transfected with an Lc-Maf expression plasmid, and quantitative RT-PCR was performed to measure the expression of endogenous *Col27a1* and *Col11a2* genes. Endogenous *Col27a1* was upregulated 6-fold by Lc-Maf overexpression, while endogenous *Col11a2* was unchanged. Finally, *in situ* hybridization and immunohistochemistry were performed in the radius and ulna of embryonic day 17 mouse forelimbs undergoing endochondral ossification. Results demonstrated that *Lc-Maf* and *Col27a1* mRNAs are coexpressed in proliferating and prehypertrophic regions, as would be predicted if Lc-Maf regulates *Col27a1* expression. Type XXVII collagen protein was also most abundant in prehypertrophic and proliferating chondrocytes. Others have shown that mice that are null for Lc-Maf and c-Maf have expanded hypertrophic regions with reduced ossification and delayed vascularization. Separate studies have indicated that *Col27a1* may serve as a scaffold for ossification and vascularization. The work presented here suggests that Lc-Maf may affect the process of endochondral ossification by participating in the regulation of *Col27a1* expression.

Introduction

Maf-family proteins belong to the basic leucine zipper (bZIP) superfamily of transcription factors; they contain a leucine zipper dimerization interface adjacent to a basic DNA-binding region [1]. However, the basic regions of the Maf family of proteins contain non-conservative amino acid substitutions at positions that are highly conserved in other bZIP proteins. Maf proteins also contain an additional DNA-binding region on the N-terminal side of the bZIP domain that is highly conserved and required for specific DNA recognition [2]. These differences result in a different tertiary structure and DNA contact interface than the canonical bZIP proteins [3]. Maf proteins often recognize longer DNA sequence elements (13–14 bp) that extend past the typical bZIP recognition element [2–4].

Individual Maf proteins can be widely expressed in diverse cell types and yet are involved in the expression of a variety of tissue-

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specific gene products both during and after development [5–9]. This characteristic appears to be due, in part, to the ability of bZIP proteins to form heterodimers with a wide variety of other transcriptional regulatory proteins. Due to the nature of dimer formation among bZIP proteins, in which both proteins independently recognize one half of the DNA recognition sequence, Maf recognition elements frequently differ from each other [2]. It has been suggested that Maf proteins acquire their ability to regulate expression of such a diversity of genes through interacting with different transcriptional regulatory proteins in the developing tissues and thereby recognizing a variety of target-specific DNA elements [1,3,10–12].

The long form of c-Maf (Lc-Maf) was identified in a yeast two-hybrid screen by its specific interaction with SOX9, a known transcriptional activator of a number of cartilage-specific genes, including those for the type II, IX, XI, and XXVII collagens [7,13–16]. Lc-Maf was shown to synergize with SOX9 to increase the expression of the cartilage-specific *Col2a1* gene in 10T1/2 cells and MC615 chondrocytes by binding within a *Col2a1* enhancer element to the short, 7-bp recognition element GGCTCTG [7]. Lc-Maf is a splice variant of c-Maf, it has a different 3′UTR region and the protein has an additional 10 amino acids at the carboxyl terminus [7,17]. Using a probe from the 3′UTR of Lc-Maf, northern hybridizations revealed RNA expression in various mouse tissues, including cartilage [7,17]. It has been demonstrated that the presence of Lc-Maf and c-Maf in cartilage plays a vital role for correct skeletal development: mice lacking these proteins exhibited decreased fetal bone length and had improper hypertrophic chondrocyte differentiation [18].

Cartilage is an important tissue that serves a vital role as the template for many bones in the developing skeleton. During the process of endochondral ossification, long bones of the body develop from a cartilage intermediate that is progressively replaced by bone. During the first phase, the mesenchyme cells differentiate into chondrocytes. Starting at the center of the cartilaginous template and progressing towards the epiphyses, these cells mature from reserve to proliferating and on to hypertrophic chondrocytes, at which point mineralization begins. At each stage of chondrocyte differentiation, the expression of extracellular matrix proteins are temporally and spatially coordinated due to a tight regulation via transcription factors, signaling molecules, hormones and local growth factors [7,19]. Precise coordination of the expression of extracellular matrix proteins, such as the cartilage-specific collagens, is essential for correct skeletal development.

Because Lc-Maf was shown to increase *Col2a1* expression, we investigated the role it might play in regulating other cartilage-specific collagen genes that are also involved with skeletal development. Specifically, we focused on the D/E enhancer element from *Col11a2* and the 27F/G enhancer element from *COL27A1*. Like the *Col2a1* enhancer, these elements are both responsive to SOX9 [13–15,20,21]. Our findings indicate that the transcription factor Lc-Maf does indeed help activate expression of *Col27a1* but not *Col11a2*.

Hjorten et al. have suggested on the basis of protein localization studies that *Col27a1* plays a role in the later stages of endochondral ossification [22]. In a separate study, MacLean et al. demonstrated that absence of both c-maf and Lc-maf causes abnormal endochondral ossification [18]. The present study suggests that Lc-Maf may affect the process of endochondral ossification by participating in the regulation of *Col27a1* expression.

Materials and methods

Plasmids

Each of the reporter constructs used in transient transfections contained four tandem copies of an enhancer element, cloned upstream of the 95-bp *Col2a1* minimal promoter and a luciferase reporter gene. Enhancer elements tested in plasmids 4×(D/Em) p95Luc and 4×(27F/Gm)p95Luc were synthesized as complementary oligonucleotides purchased from Invitrogen (Carlsbad, CA). Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before annealing. The plasmid p89Col2a1Bs was used to multimerize the enhancer elements to four tandem copies before transferring them, along with the *Col2a1* minimal promoter, into the luciferase reporter vector pLuc4 as previously described [13,23]. The other luciferase reporter plasmids were made previously [13–15]. The p89Col2a1Bs plasmid was a gift from Dr. Veronique Lefebvre of the Cleveland Clinic in Cleveland, Ohio. The Lc-Maf expression vector pcDNA3.1-Lc-Maf was a gift from Dr. Wendong Huang of the Baylor College of Medicine in Houston, Texas.

Transient transfections

Rat chondrosarcoma (RCS) cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and 10% bovine growth serum. Every 3–4 days the cells were passaged using a 0.25% trypsin-1 mM EDTA solution.

Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's protocol. A total of 2 μg of DNA was used to transfect each 10-cm^2 well. Each reaction included 1.0 μg of the luciferase reporter plasmid, 0.5 μg of pSV-β-galactosidase as an internal control for transfection efficiency, and 0.5 μg of either pcDNA3.1-Lc-Maf or the empty pcDNA3.1 vector. β-galactosidase levels were measured using the Galacto-Light/Plus system (Tropix, Bedford, MA) following the manufacturer's protocol. Luciferase levels were measured using the Luciferase Assay Reagent (Promega. Madison, WI) following the manufacturer's protocol. Transfection data is reported as Relative Luciferase Units (luciferase units per β-galactosidase unit) ± standard error, normalized to the activity of the appropriate enhancer/reporter gene construct within each experiment. Each graph represents at least three independent experiments, each performed in triplicate. Results were analyzed for statistical significance using Student's *t*-test.

In vitro transcription/translation

Lc-Maf protein was synthesized using the Single Tube Protein System 3, T7 kit (Novagen, San Diego, CA) according to manufacturer's instruction using the Lc-Maf expression plasmid, pcDNA3.1-Lc-Maf. To verify that transcription and translation occurred as expected, [^{35}S]methionine-labeled Lc-Maf was visualized on SDS-PAGE.

Electrophoretic mobility shift assay (EMSA)

Wild-type and mutant *COL27A1* 27F/G probes were prepared by annealing complementary oligonucleotides synthesized by Invi-