Molecular Cloning and Characterization of a Novel Gene, CORS26, Encoding a Putative Secretory Protein and Its Possible Involvement in Skeletal Development*

We cloned a novel mouse cDNA, CORS26 (collagenous repeat-containing sequence of 26-kDa protein), encoding a secretory protein by suppression subtractive hybridization between transforming growth factor-β1-treated and untreated C3H10T1/2 cells. The deduced amino acid sequence of CORS26 consists of 246 amino acids with a secretory signal peptide and contains a collagenous region (Gly-X-Y repeats) at the NH2 terminus and a complement factor C1q globular domain at the COOH terminus. CORS26 is structurally similar to C1q and to adipocyte-specific protein Acrp30. Transfection analysis suggested that CORS26 is a secretory protein. Northern blot analysis revealed that CORS26 mRNA was present at high levels in rib growth plate cartilage and at moderate levels in kidney of adult mice. CORS26 mRNA was not detected in NIH3T3 cells, BALB/3T3 cells, C3H10T1/2 cells, or osteoblastic MC3T3-E1 cells by reverse transcription-polymerase chain reaction analysis. In situ hybridization of mouse embryos between 13 and 15 days postcoitus revealed relatively high levels of CORS26 mRNA in condensed prechondrocytic cells of cartilage primordia and developing cartilages. However, CORS26 mRNA were undetectable in mature chondrocytes. Furthermore, overexpression of CORS26 enhanced the growth of C3H10T1/2 cells in vitro. The present findings suggest that the CORS26 gene may play an important role in skeletal development.

In skeletal development in vertebrates, the formation of chondrocytes from undifferentiated mesenchymal cells is one of the important processes, but the molecular mechanisms are not well understood. Identifying the genes underlying the induction of chondrocyte differentiation will provide powerful tools for understanding skeletal development. The induction of chondrogenesis has been extensively studied in vitro using primary cells and clonal cell lines from a variety of sources (1–4). The mouse embryonic fibroblast cell line, C3H10T1/2, are multipotential cells and have been induced to undergo differentiation into myocytes, adipocytes, osteoblasts, and chondrocytes under specific culture conditions and treatments (5–8). The frequency of chondrogenic conversion in C3H10T1/2 cells was much lower and irregular compared with other types of conversion (5, 8), but it was recently reported that the induction of chondrogenesis and the formation of spheroids in C3H10T1/2 cells preferentially occurred when treated with transforming growth factor (TGF)-β1 (9), bone morphogenetic protein-2 (10), or a combination of osteoinductive bone proteins (11) in high-density micromass cultures. The formation of the spheroids resembled the condensation of mesenchymal cells seen in precartilage. Thus, C3H10T1/2 cells in high-density micromass cultures are suited for studying the molecular mechanisms involved in skeletal development.

In the present study, to help clarify the mechanism for skeletal development, mRNAs expressed in TGF-β1-treated C3H10T1/2 cells were subtracted with those in untreated C3H10T1/2 cells using the suppression subtractive hybridization (SSH) technique (12), and we isolated a novel gene, CORS26 (collagenous repeat-containing sequence of 26-kDa protein). Sequence analysis revealed that CORS26 possesses a collagenous structure at the NH2 terminus and a complement factor C1q globular domain at the COOH terminus. Due to the structural similarity between CORS26 and subunits of complement factor C1q, this novel protein is thought to be a member of the C1q-related protein family. The presence of the signal peptide, plus the hydrophilic nature of CORS26, suggests that CORS26 is a secretory protein. Indeed, the CORS26 protein was secreted from COS-7 cells by transient transfection analysis. CORS26 mRNA is specifically expressed in cartilage and kidney in the adult mouse. Moreover, the expression of this novel gene was observed in cartilage primordium or developing cartilage in embryonal mouse tissues in vivo. Thus, a possible secretory protein encoded by the CORS26 gene may be one of the important signaling molecules produced by prechondrocytic mesenchymal cells or early chondrocytes during skeletal development.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—The mouse cell line, C3H10T1/2, was obtained from the RIKEN Cell Bank (Tsukuba, Japan), and micromass cultures were performed as described previously (9). In brief, trypsinized cells were suspended in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc.), penicillin (50 units/ml), and streptomycin (50 µg/ml).

1 The abbreviations used are: TGF, transforming growth factor; SSH, suppression subtractive hybridization; FBS, fetal bovine serum; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair(s); kb, kilobase pair(s); p.c., post coitus; RACE, rapid amplification of cDNA end(s); DMEM, Dulbecco’s modified Eagle’s medium; CRF, complement protein C1q-related factor; Acrp, adipocyte complement-related protein; TNF, tumor necrosis factor; TNFR, TNF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank**

**EBI Data Bank with accession number(s) AF246265.**

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ml) at a concentration of 10^7 cells/ml, and then a 10-μl drop of this cell suspension was placed in the center of a 24-well dish at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were allowed to adhere to the dish for 3 h, and the culture was flooded with 1 ml of medium. After 24-h incubation, 1 ng/ml TGF-β1 was added to the culture, and incubation was continued at 37 °C for 5 h. This condition was used for evaluating for accuracy and was used for in vitro transcription and translation.

**In Vitro Transcription and Translation**—The TNT T7 quick coupled transcription/translation system (Promega) was used to transcribe and translate the full-length CORS26 cDNA construct in the presence of [35S]methionine (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Autoradiography was performed on a 12.5% SDS-polyacrylamide gel. The gel was treated with Enlightening (PerkinElmer Life Sciences), dried, and autoradiographed.

**RT-PCR Analysis**—Total RNAs isolated from various cell lines were digested with RNase-free DNase I (Promega) at 37 °C for 15 min. Then the denatured 1 μg of total RNA was reverse-transcribed in 20 μl of a reaction mixture containing 5 × RT buffer, 10 mM dithiothreitol, 0.5 mM each of dNTP, 500 ng of oligo(dT)_18 primers, 10 units of RNase inhibitor, and 200 units of Moloney murine leukemia virus reverse transcriptase (Wako Pure Chemicals, Osaka, Japan) at 37 °C for 1 h and subsequently heated at 70 °C for 10 min. After the RT reaction, PCR was carried out in a total volume of 25 μl of a mixture containing 1 μl of RT samples, 10 μM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 μM each of dNTP, 1 unit of AmpliTaq Gold DNA polymerase (PerkinElmer Life Sciences). Twenty-five cycles of denaturation (94 °C, 30 s), annealing (54 °C, 30 s), and extension (72 °C, 45 s) were carried out in a DNA thermal cycler (PerkinElmer Life Sciences). The primer sequences for CORS26 were 5'-GAAACATGCGAAGATGGG-3' and 5'-TCTGTAAGGATGGA-3' AATA 5'. Aliquots (10 μl) of the PCR products were electrophoresed in 1.5% agarose gel, and the gel was stained with ethidium bromide and photographed under UV light. The amplified 300-bp PCR products were cloned into pGEM-T Easy vector.

**In Situ Hybridization**—One microgram of recombinant pGEM-T Easy vector containing the 300-bp fragment located at the coding region (nucleotides 305–604) of CORS26 was used as a template. Plasmids were linearized with SpeI to prepare the antisense riboprobe and XhoI to prepare the sense riboprobe. In *in vitro* transcription was performed with 32P-UTP (Amersham Pharmacia Biotech) using T7 and SP6 RNA polymerase (Promega). Unincorporated labels were removed by ethanol precipitation, and the counts/min were determined on a scintillation counter.

**Allografts** were obtained from ICR mice. In *in situ* hybridizations were carried out as described previously (14) with modifications. In brief, mouse embryos were fixed with 4% paraformaldehyde and embedded in paraffin. The 7-μm-thick sections were pretreated with proteinase K and HCl and acetylated. Hybridization was performed with riboprobes (1 × 10⁶ cpm per slide) at 53 °C overnight. After hybridization, the sections were washed with 5 × SSC containing 10 mM dithiothreitol and 1 × SSC containing 0.1% SDS, followed by a final rinse at 72 °C in 1 × SSC. The sections were dehydrated in a graded series of ethanol, air-dried, coated with NTB-2 emulsion (Eastman Kodak Co.) and exposed for 2–3 weeks. Microphotographs were taken using both light- and dark-field optics.

**Transient Transfection with FLAG-tagged CORS26 and Immunoblotting**—A CORS26 cDNA was tagged with a FLAG epitope at the COOH terminus by PCR using oligonucleotides 5'-TTTCGGCGACCGAGG-3' and 5'-TCACCGAGTCTCTTCTGA-3'. The sequence for a FLAG tag was underlined. The amplification conditions were 80 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min and a final incubation at 72 °C for 5 min. The PCR product was cloned into pGEM-T Easy vector, and the insert was excised with EcoRI and reinserted into the EcoRI site of pcDNAs 1.1 + vector (Invitrogen). The orientation and nucleotide sequence of pcDNAs1.1+CORS26-FLAG was confirmed by automated ABI 373 sequencer. The pcDNAs1.1+CORS26-FLAG construct was transfected into COS-7 cells (RIKEN Cell Bank) using the SuperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. A pcDNAs 1.1 + vector was transfected in parallel as a negative control.

**Transfections** were performed on cells seeded into six-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS 24 h before use at a density of 3 × 10⁵ cells/well.
Cells were typically transfected with 2 μg of DNA, incubated with the DNA suspension for 3 h, and replenished with fresh medium. Then, 48 h after transfection, conditioned medium was collected, and cells were lysed with TNE buffer (10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40). Protein from the conditioned medium was concentrated for 1 h with a Centricon-10 concentrator (Millipore, Bedford, MA). The proteins from the medium and cell extracts were separated on a 12.5% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane in a Trans-Blot semidry electrophoretic transfer cell (Bio-Rad) and immunoblotted with anti-FLAG M2 monoclonal antibody (Sigma) diluted 1:500. Immunocomplexes were detected with a secondary antibody conjugated to peroxidase (Dako, Glostrup, Denmark) and visualized with ECL reagent (Amersham Pharmacia Biotech).

**RESULTS**

Molecular Cloning and Sequence Analysis of the CORS26 cDNA—To identify genes specifically expressed during skeletal development, the SSH technique was utilized using the mRNA extracted from C3H10T1/2 cells with or without TGF-β1 treatment, and we selected several clones specifically up-regulated by TGF-β1 treatment. We determined the sequence of the isolated partial cDNA clones and carried out homology searches in the GenBank™ using BLAST2. Among all clones that had no significant homology with any known genes in the nucleotide sequence data bases, a 380-bp cDNA clone (clone 129) showed a marked induction by TGF-β1 treatment (Fig. 1). We, therefore, focused further analysis on this clone. To obtain a full-length sequence,

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**Fig. 1.** Northern blot analysis confirming differential gene expression for clone 129. Total RNA obtained from C3H10T1/2 cells without or with TGF-β1 treatment was electrophoresed. The blot was stripped and reprobed with a GAPDH probe as the loading control.

**Fig. 2.** Nucleotide sequence and the predicted amino acid sequence of CORS26. The triangle indicates the putative signal peptide cleavage site. The position of collagen-like domains are circled. Two separate consensus polyadenylation signals are underlined.

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we carried out the 5′- and 3′-RACE using a TGF-β1-treated C3H10T1/2-derived cDNA. The full nucleic acid sequence obtained (1,879 bp) and the deduced amino acid sequence are shown in Fig. 2. The full length of 1,879 bp obtained was in good agreement with the transcript size of ~2.0 kb estimated on Northern blots. An open reading frame containing a coding region of CORS26 was inserted into the EcoRI-EcoRV sites of pcDNA3.1(+) vector. C3H10T1/2 cells were seeded into 35-mm culture dishes in DMEM supplemented with 10% FBS at a density of 3 \times 10^4 cells per dish. Twenty-four hours later, the cells were transfected with 2.5 μg of either control pcDNA3.1 vector or pcDNA3.1/CORS26 using the SuperFect Transfection Reagent (Qiagen) according to the manufacturer’s instruction. The cells were incubated for 48 h and then trypsinized and seeded at a 1:20 ratio in 100-mm culture dishes in DMEM containing 10% FBS. Sixteen hours later, the cells were switched to a selective medium containing 500 μg/ml G418 (Promega). After 4 weeks of culture in the selective medium, clonal isolates were expanded, and expression of CORS26 mRNA was verified by RT-PCR as described above. To analyze the growth of C3H10T1/2 cells transfected with pcDNA3.1/CORS26, the transfectants were seeded in 24-well culture plates at a density of 1 \times 10^4 cells/well and cultured with DMEM supplemented with 10% FBS. The number of cells were counted with a hemocytometer at the indicated days.
The translation products were separated on 12.5% SDS-polyacrylamide gel and visualized by autoradiography. An ~26-kDa protein was generated. The protein molecular mass is indicated by kilodaltons on the left side. B, hydrophobicity plot of CORS26 protein. Positive values indicate hydrophobic, and negative values indicate hydrophilic regions.

It was recently demonstrated that the mouse embryonic mesenchymal cell line, C3H10T1/2, when cultured at high density,

directed protein also contained other putative functional sites, phosphorylation sites for protein kinase C (at amino acid positions 152–154) and casein kinase II (at amino acid positions 77–80 and 138–141) and several N-myristoylation sites.

Expression of CORS26 mRNA in the Adult Mouse Tissues—We examined the expression of CORS26 mRNA in various adult mouse tissues by Northern blot analysis (Fig. 6). CORS26 cDNA hybridized to double transcripts of 2.3 and 2.0 kb. The CORS26 mRNA was expressed in rib growth plate cartilage and kidney. CORS26 mRNA was not detected in other tissues. In growth plate cartilage, the hybridization signal at 2.0 kb was stronger than the signal at 2.3 kb, while, in kidney, the signal at 2.3 kb was stronger than the signal at 2.0 kb. The 2.0-kb size of the transcripts agrees approximately with the length of the cloned CORS26 cDNA.

Expression of CORS26 mRNA in Various Fibroblastic or Osteoblastic Cell Lines—By RT-PCR analysis, the expression of CORS26 mRNA was not detectable in NIH3T3 cells, BALB/3T3 cells, C3H10T1/2 cells, and MC3T3-E1 cells (Fig. 7).

In Situ Hybridization of CORS26 in the Embryonal Mouse Tissues—To examine the expression of CORS26 during embryonic development in vivo, we performed in situ hybridization using embryonal mouse serial sections at 13–15 days p.c. CORS26 transcripts were localized in regions of cartilage primordium of occipital bone and that of the vertebral body of a 13-day-p.c. embryo (Fig. 8, A–C and F–H). In Meckel’s cartilage, high levels of CORS26 mRNAs were seen in a 15-day-p.c. embryo (Fig. 8, D and I). In the cartilage primordium of digital bone of a 14-day-p.c. embryo, CORS26 was expressed in prechondrocytes, but not in mature chondrocytes (Fig. 8, E and J). These experiments showed that CORS26 mRNA was present at relatively high concentrations in precartilaginous primordia and developing cartilages. No specific signal was detected above background levels by sense riboprobes as controls.

Growth of CORS26 Transfected Cells in Vitro—We generated C3H10T1/2 cells stably transfected with the mammalian expression vector containing CORS26. The expression of CORS26 mRNA in the transfec tant was verified by RT-PCR analysis (data not shown). The growth of CORS26 transfec tants was significantly enhanced when compared with that of control cells. Moreover, saturation densities of CORS26 transfec tants were higher than that of control cells (Fig. 9).

DISCUSSION

During skeletal development, condensation of multipotential mesenchymal cells to differentiate toward the various cell types is an important process. One such process is the formation of chondrocytes from undifferentiated mesenchymal cells. It was recently demonstrated that the mouse embryonic mesenchymal cell line, C3H10T1/2, when cultured at high density,
is induced to undergo chondrogenic differentiation by TGF-β1. C3H10T1/2 cells cultured under this condition form a three-dimensional spheroid structure, and the morphology of the cells in the spheroid resembled that of the cells seen in precartilage condensations (9). The formation of the spheroid in vitro mimics the condensation event of chondrogenesis in vivo.

In the present study, we demonstrated isolation of a novel gene, CORS26, encoding a secretory protein of 246 amino acids using a suppression subtractive hybridization technique between TGF-β1-treated and untreated C3H10T1/2 cells cultured at high density. Sequence analysis reveals that CORS26 protein has a hydrophobic signal peptide at the NH2 terminus and lacks a putative transmembrane domain. The presence of a putative signal peptide suggests that CORS26 enters the secretory pathway. Actually, CORS26 was secreted from COS-7 cells after transient transfection of a FLAG-tagged CORS26 expression vector. The deduced amino acid sequence of CORS26 displays structural similarity to several C1q related proteins, such as C1q A, B, and C chains, Acrp30, and CRF, containing the collagenous repeats (Gly-X-Y) at the NH2 terminus and the globular domain at the COOH terminus. This suggested that CORS26 may belong to the C1q family proteins.

C1q family proteins are known to homo- or hetero-oligomerize via the collagenous structures, suggesting that CORS26 might form oligomers with itself or other proteins. The COOH-terminal region of the protein contains three potential phosphorylation sites. Consequently, this molecule is a potential target for protein phosphorylation via protein kinase C and casein kinase II. In addition, a cysteine residue in the globular region of the C1q B and C chains plays an important role in the formation of disulfide bonds with IgG (27) and the stabilization of the triplex strands in the collagenous domain (24). These cysteine residues are replaced by other residues in CORS26 similar to Acrp30 and CRF.

Recently, it was reported that the crystal structure of Acrp30, a member of the C1q family of proteins, showed homology with that of the tumor necrosis factor (TNF) family proteins. Moreover, TNFs (28) and C1q proteins have similar gene structure in the globular domains (29). These similarities suggest an evolutionary link between the C1q-like proteins and TNFs and establish a C1q/TNF molecular superfamily. It has been reported that TNFs have monospecific receptors (TNFRs) (30). Since CORS26 and C1q share significant similarities in the structure of the collagenous domain, which has been shown to be important for ligand-receptor interaction (13), it is possible that CORS26 might signal through the TNFRs or TNFR-like receptors.

Both Northern blot analysis and RT-PCR analysis showed a unique pattern of gene expression of CORS26 in various tissues and cell lines. CORS26 mRNA expression was found in rib...
Signals were detected in the prechondrocytes of developing digits of a 13-day-p.c. embryo. In situ hybridization of mouse embryos 13–15 days p.c. showed that CORS26 was highly expressed in precartilaginous primordia and Meckel’s cartilage. In each case, CORS26 signals were detected in the prechondrocytes of developing digits of a 14-day-p.c. embryo (E and J). Bar, 1,500 μm in A, 200 μm in B, C, and 100 μm in D and E.

**Fig. 8. In situ hybridization analysis of CORS26 expression in embryonal mouse tissues.** Bright-field (A–E) and dark-field (F–J) microphotographs of the sections hybridized to CORS26 antisense riboprobes are shown. The CORS26 signal is shown in the cartilage primordium of occipital bone (A and F), of lumbar vertebral body (B and G), and sacral vertebral body (C and H) of a 13-day-p.c. embryo. D and I show the signal in Meckel’s cartilage of a 15-day-p.c. embryo. CORS26 signals were detected in the prechondrocytes of developing digits of a 14-day-p.c. embryo (E and J). Bar, 1,500 μm in A, 200 μm in B, C, and 100 μm in D and E.

Growth of CORS26 transfectants in vitro. C3H10T1/2-CORS26 (closed circle) or C3H10T1/2-vector (open circle) were seeded into 24-well culture plates at a density of 1 × 10^5 cells/well. The number of cells were counted in triplicate at the indicated days. Results are shown as mean ± S.D.

Although the function of CORS26 in vivo is not clear, one possible function is the local regulation of mesenchymal condensations as secreted autocrine/paracrine factors during an early stage of skeletal development. Further analysis of CORS26 will lead to a better understanding of the molecular mechanism in skeletal development.

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