Ucma, a Novel Secreted Cartilage-specific Protein with Implications in Osteogenesis*§

Received for publication, April 2, 2007, and in revised form, December 4, 2007 Published, JBC Papers in Press, December 21, 2007, DOI 10.1074/jbc.M707922200

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Here we report on the structure, expression, and function of a novel cartilage-specific gene coding for a 17-kDa small, highly charged, and secreted protein that we termed Ucma (unique cartilage matrix-associated protein). The protein is processed by a furin-like protease into an N-terminal peptide of 37 amino acids and a C-terminal fragment (Ucma-C) of 74 amino acids. Ucma is highly conserved between mouse, rat, human, dog, clawed frog, and zebrafish, but has no homology to other known proteins. Remarkable are 1–2 tyrosine sulfate residues/molecule and dense clusters of acidic and basic residues in the C-terminal part. In the developing mouse skeleton Ucma mRNA is expressed in resting chondrocytes in the distal and peripheral zones of epiphyseal and vertebral cartilage. Ucma is secreted into the extracellular matrix as an uncleaved precursor and shows the same restricted distribution pattern in cartilage as Ucma mRNA. In contrast, antibodies prepared against the processed C-terminal fragment located Ucma-C in the entire cartilage matrix, indicating that it either diffuses or is retained until chondrocytes reach hypertrophy. During differentiation of an MC615 chondrocyte subclone in vitro, Ucma expression parallels largely the expression of collagen II and decreases with maturation toward hypertrophic cells. Recombinant Ucma-C does not affect expression of chondrocyte-specific genes or proliferation of chondrocytes, but interferes with osteogenic differentiation of primary osteoblasts, mesenchymal stem cells, and MC3T3-E1 pre-osteoblasts. These findings suggest that Ucma may be involved in the negative control of osteogenic differentiation of osteochondrogenic precursor cells in peripheral zones of fetal cartilage and at the cartilage-bone interface.

Elucidation of molecular mechanisms underlying chondrocyte differentiation is not only important for our understanding of skeletal development, but also of particular interest for our knowledge on the behavior of chondrocytes following articular cartilage damage during cartilage repair and treatment of degenerative cartilage diseases. Initial steps of chondrogenesis, i.e. the formation of a cartilage blastema from limb bud mesenchymal cells, include cell condensation and onset of chondrocyte differentiation marked by the expression of cartilage-specific matrix proteins such as aggrecan, collagen II, IX, and XI and others (1, 2). These events are regulated by the orchestrated action of several growth factors including BMPs, Wnt factors, FGFs, and the transcription factors Sox5, 6, and 9 (3, 4). Further steps of chondrocyte growth, maturation, and replacement by bone in the growth plate of long bones, ribs, and vertebrae during endochondral ossification can be defined by the stepwise onset or decline of differentially expressed genes: collagen II and Sox9 for resting and proliferating, FGF3 for proliferating and prehypertrophic, Ihh and PTHrP receptor for prehypertrophic, collagen X for hypertrophic, and Runx2, osteocalcin, and MMP13 for late hypertrophic chondrocytes (2, 5–7). Similar to chondrogenesis, the steps of chondrocyte differentiation during endochondral ossification are regulated in a complex manner by BMPs, Wnt factors, and FGFs. In addition, Ihh/PTHrP, HIF-1α, thyroxine, and others are important regulators of this process (2, 7).

Little is known, however, on the early differentiation events occurring in the distal, so-called “resting” zone of epiphyseal cartilage. In a search for further chondrocyte specific genes, we identified a novel, highly charged extracellular protein that is abundantly expressed in the upper immature zone of fetal and juvenile epiphyseal cartilage. The gene, termed Ucma, was discovered in a screen for differentially expressed genes in retinoic acid-treated mouse chondrocytes. Independently, Ucma was also identified in a human chondrocyte EST screen for candidate genes of skeletal dysplasias (8). Here we report on the transient expression of Ucma in the developing mouse skeleton between day E13.5 of embryonic development and 5 months of postnatal development. Ucma is predominantly expressed in distal peripheral, non-articulating zones of fetal epiphyses and vertebrae, but not by chondrocytes of the hypertrophic zone. Ucma contains tyrosine sulfate and is cleaved by a furin-like
protease into N- and C-terminal fragments of 37 and 74 amino acid residues, respectively. With specific antibodies we show that the distribution of the unprocessed form of Ucma is restricted to sites of Ucma mRNA expression, while the C-terminal fragment Ucma-C is found in almost the entire cartilage matrix. During differentiation of MC615 chondrocytes in vitro Ucma expression largely parallels the expression of collagen II, and declines with onset of hypertrophy. Furthermore, we provide evidence that recombinant Ucma-C protein suppresses osteogenic differentiation of preosteoblasts in vitro. Therefore, we postulate that this protein may have a transient function in the formation and stabilization of the juvenile cartilage matrix of epiphyseal, vertebral, and rib cartilage by suppressing osteogenic differentiation of precursor cells present in peripheral zones and perichondrium of fetal cartilage.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Skeletal Cells—MC615 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)2/Ham’s F12 supplemented with 10% fetal calf serum (FCS) and 2% fetal calf serum (FCS) and passaged before reaching confluency as outlined before (9, 10). MC3T3-E1 cells were maintained in DMEM/Ham’s F12 with 10% FCS and subcultured when reaching confluence. To obtain clones of MC615 cells with unique differentiation states individual subclones of this cell line were isolated by limiting dilution. Chondrogenic differentiation of one of these subclones (4C6) in vitro was achieved within 3–5 weeks in normal growth medium.

Osteogenic differentiation of MC3T3-E1 preosteoblasts was achieved by prolonged confluent culture in differentiation medium (DMEM/Ham’s F12, 10% FCS, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate) as previously described (12). Primary mouse calvarial cells were isolated from 3–6-day-old mice as outlined earlier (11).

Human mesenchymal stem cells (hMSCs) were isolated from fresh umbilical cord blood from healthy donors. After expansion osteogenic differentiation of hMSCs was achieved by prolonged culture in differentiation medium (60% DMEM-LG (Invitrogen), 40% MCDB-201, 1× insulin-transferrin- selenium, 1× linoleic-acid-bovine-serum-albumin, 10−5 M dexamethasone, 10−4 M ascorbic acid 2-phosphate, 10 ng/mL EGF (all from Sigma-Aldrich), 10 ng/mL PDGF-BB (R&D Systems), 100 units of penicillin, 1000 units of streptomycin (Invitrogen) and 2% fetal calf serum) as reported earlier (13). For isolation of primary murine chondrocytes rib cages from newborn mice were treated with 1 mg/mL trypsin in serum-free DMEM/Ham’s F12 medium (PAA) at 37 °C for 45 min followed by digestion with collagenase P (Roche Applied Science, Mannheim, Germany) (2 mg/mL in DMEM/Ham’s F12, 10% FCS, 2 h at 37 °C) (10). Rib chondrocytes were seeded in DMEM/Ham’s F12 containing 10% FCS at densities of 1 × 10^4 to 2.5 × 10^4 cells/cm². Bovine epiphyseal chondrocytes were isolated from the growth plates of fetal calves (about 4–6 months of gestation) and separated into distinct fractions by centrifugation through a linear Percoll gradient as previously described (10, 14). For BMP stimulation of cells normal growth medium was replaced by DMEM/Ham’s F12 medium without or with 1% FCS supplemented with recombinant BMP-2 at the indicated concentrations for 24 h or 48 h.

Detection of the Transcription Start Site of the Ucma Gene—The transcription start site of Ucma was detected by rapid amplification of 5′-cDNA ends (5′-RACE) using the GeneRacer™ kit (Invitrogen) according to the manufacturer’s instructions. For this purpose poly(A)+ RNA was obtained from primary murine rib chondrocytes using the Oligotex® mRNA Mini kit (Qiagen, Hilden, Germany). 150 ng poly(A)+ RNA was used in a 5′ GeneRacer assay with gene specific primers 5′-GGG GTG TTG AGA GTG AGG GA-3′ and 5′-CTA TCT TTC TCT TGC TCG TAA ACG AGT T-3′ for primary and nested PCR reactions, respectively. The product of the nested PCR reaction was cloned into pCR4-TOPO (Invitrogen, Karlsruhe, Germany) by TOPO-TA cloning and 12 individual clones were sequenced to reveal the 5′ cDNA end representing the transcription start site of Ucma.

Analysis of mRNA Expression—Total RNA for expression analyses and cloning was isolated using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. To avoid any contamination with genomic DNA the optional DNase step was included. Reverse transcription for (real-time) RT-PCR was performed using SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR reactions were carried out as previously reported using the TaqPCR Core Kit (Qiagen) and gene-specific primers (11).

Real-time RT-PCR was performed as described previously (10). Cyclophilin A and/or β-actin were used to standardize the total amount of cDNA in real-time PCR approaches. The following primers were used for detection of respective mRNA levels by (real-time) RT-PCR: murine Ucma: 5′-GGT TCT GGG GTT GCT CGT TA-3′ (sense primer); 5′-GAG GAA ATT GGA GGC ATC AG-3′ (antisense primer); bovine Ucma: 5′-CTC AGC CTT TCC AAA GAT GG-3′ (sense primer); 5′-GGG TAC AGG CCG TCG TAA T-3′ (antisense primer); murine Runx2: 5′-ATA CCC CCT GCG TCT CTG TT-3′ (sense primer); 5′-AGG TTG GAG GCA CAC ATA GG-3′ (antisense primer); murine Col10a1: 5′-CAT AAA GGG CCC ACT TGC TA-3′ (sense primer); 5′-CAG GAA TGC CTT GCT CTC CT-3′ (antisense primer); murine β-actin: 5′-AGA GGG AAA TCG TGC GTG AC-3′ (sense primer) and 5′-CAA TAG TGA TGA CCT GGC GT-3′ (antisense primer); murine cyclophilin A: 5′-CCA CCG TGT TCT TCG ACA T-3′ (sense primer) and 5′-CAG TGC TCA GAG CTC GAA AG-3′ (antisense primer).

For RT-PCR control reactions sequences of murine and bovine glyceraldehyde-3-phosphate dehydrogenase (Gapdh) primers were adopted from PCR-Select cDNA Subtraction Kit (BD Clontech Germany, Heidelberg, Germany) and Schmid et al. (10), respectively.

2 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; RACE, rapid amplification of cDNA ends; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; hMSCs, human mesenchymal stem cells; RT, reverse transcription; MS, mass spectrometry; AA, ascorbic acid; β-GP, β-glycerophosphate; AP, alkaline phosphatase.
Ucma, a Novel Cartilage-specific Secreted Protein

Northern blot analyses were carried out as described before (12). A cDNA probe for Ucma was generated by RT-PCR using the following cDNA-specific primers: 5'-CCAGTGCGCAT-TATGATGCC-3' (sense primer); 5'-TGAAAGTGTCTAT-CAGATGGGTG-3' (antisense primer). For detection of collagen type II transcripts a cDNA probe encompassing nucleotides 4464 to 4858 of the procollagen, type II, alpha 1 (Col2a1), mRNA sequence (Genbank, NM_031163.2) was employed and Gapdh transcripts were hybridized with a probe previously described (12).

RNA in situ hybridization on paraffin sections of mouse tissues with digoxigenin-labeled antisense riboprobes for Ucma and collagens α1(II) and α1(X) was carried out as reported before (10). Specific cDNA fragments for Ucma antisense riboprobes including nucleotides 451–784 or 131–544 of the murine Ucma mRNA sequence were obtained by RT-PCR and cloned into the pCRII-TOPO vector.

Generation of Polyclonal Rabbit Antibodies (TAGL-1 and UCMA-1) Directed against Ucma—For preparation of antibodies against unprocessed Ucma, a 17-amino acid peptide optimized for rabbit immune response (MSNFLKKRRGKRSPKSRD) and overlapping the furin cleavage site (arrowhead) was conjugated to KLH. A rabbit was injected subcutaneously with 0.4 mg of Ucma-KLH peptide in complete Freund’s adjuvant followed by four booster injections in incomplete adjuvant. The antisera were purified by affinity chromatography with a HiTrap NHS-activated HP column (GE Healthcare, Sweden) with Ucma peptides and the resulting purified antibody was termed TAGL-1. Rabbit antisera against cleaved and uncleaved forms of Ucma were generated by David Biotechnologie GmbH (Regensburg, Germany) using purified recombinant KLH-conjugated Ucma-C for immunization. Antibodies were purified by affinity chromatography with recombinant Ucma-C as described above and termed UCMA-1.

Analysis of Protein Expression—For Western blot analysis, protein samples were resolved by SDS-polyacrylamide gel electrophoresis through denaturing 15% polyacrylamide gels as reported before (12, 15). After transfer onto nitrocellulose membranes protein immunodetection was carried out as described previously (10). Recombinant Ucma protein was detected with a mouse monoclonal anti-FLAG antibody (1:1000; Sigma). Endogenous Ucma protein was visualized with a mouse monoclonal anti-FLAG antibody. As loading control β-actin levels were determined on the same blot as Ucma using an anti-β-actin antibody (Sigma).

Immunohistochemistry was performed as described before (10). Briefly, paraffin sections were rehydrated and pretreated with testicular hyaluronidase (2 mg/ml) for 1 h at 37 °C. After washing with TBS and blocking with 5% bovine serum albumin sections were incubated with TAGL-1 antibody (1:1000) overnight at 4 °C or for 2 h at room temperature. Bound antibody was visualized using the Link-Label IHC Detection System (Biogenex, San Ramon, CA) with biotinylated anti rabbit Ig, avidin-coupled alkaline phosphatase, and Fast Red tablets containing levamisole (Sigma-Aldrich) according to the manufacturer’s instructions.

For postembedding immunogold labeling, tissue specimens were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 to 5 h at 4 °C. Specimens were dehydrated serially to 70% ethanol at −20 °C and embedded in resin (LR White; Electron Microscopy Sciences). Ultrathin sections were successively incubated in Tris-buffered saline (TBS), 1 mg/ml testicular hyaluronidase (pH 7.0), 0.05 m glycine in TBS, 0.5% ovalbumin, and 0.5% fish gelatin in TBS, TAGL-1 antibody diluted 1:50 in TBS-ovalbumin overnight at 4 °C, and finally in 10 nm gold-conjugated secondary antibody (BioCell, Cardiff, Wales, UK) diluted 1:30 in TBS-ovalbumin for 1 h. After rinsing, the sections were stained with uranyl acetate and examined with a transmission electron microscope (906E, Leo, Oberkochen, Germany). In negative control samples, the primary antibody was replaced by PBS or equimolar concentrations of nonimmune rabbit IgG.

Preparation of Recombinant Ucma—For episcopal expression of recombinant Ucma the coding sequence of murine Ucma including the signal peptide and C-terminally fused His and FLAG tags was cloned into the pCEP-Pu vector. This construct was stably transfected into HEK293EBNA cells. Episomally expressed Ucma(His)6FLAG was collected from serum-free culture medium, concentrated by ultrafiltration on a YM10 Amicon membrane and purified by affinity chromatography on nickel-nitriolactic acid Sepharose (Qiagen). Purity, integrity, and size of purified protein were tested by SDS-PAGE, Coomassie Blue staining, and Western blotting using an anti-FLAG antibody. The peptide sequence was determined by N-terminal sequencing on an automated amino acid sequencer (Applied Biosystems).

Mass Spectrometry—Ucma protein (250 μg/ml) was diluted 1:10 using either 0.2% formic acid for intact protein analysis or 25 mM ammonium bicarbonate pH 7.8 for trypsin (Promega) digestion. 10 ng of trypsin was used (1:125), and the digestion was performed overnight at 37 °C. Intact protein samples were mixed in 1:1 ratio with matrix (10 mg/ml sinapinic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) and then applied (1–2 μl) onto the sample target plate. Digested samples were purified and concentrated using reversed phase tips, homemade Stop and Go extraction tips (16). The peptides retained from samples for MALDI-TOF MS were eluted onto dried matrix (2,5-dihydroxybenzoic acid) spots on Anchorchip™ target plates (Bruker) using 1–2 μl 50% acetonitrile, 0.1% formic acid. Samples for electrospray ionization (ESI)-MS were eluted using 10 μl 50% acetonitrile, 0.1% formic acid into autosampler glass vials (Qsert, Waters). The organic solvent was evaporated, and replaced with 0.1% formic acid.

The MALDI-TOF MS analyses were done with a Bruker Reflex III instrument run in linear mode with delayed extraction and an acceleration voltage of 20 kV. The polarity was switched between positive and negative ion mode and 50–100 spectra were summed. Trypsin autolysis peaks at 842.5100 m/z and 2211.1046 m/z were used for internal calibration in positive ion mode. The electrospray MS analysis used a Bruker Esquire-HCT ion trap instrument equipped with an Ultimate HPLC system (LC Packings) with a Pepmap™ nano-precolumn (LC
Packings, C18, 300 μm i.d. and 5 mm long) and an Atlantis™
analytical column (Waters, C18, 3 μm particles, 150 mm × 150
μm). Samples (1–10 μl) were loaded onto the precolumn and
rinsed with 0.1% formic acid for 5 min. Bound peptides were
eluted using a gradient consisting of solution A (3% acetonitrile,
0.1% formic acid) and solution B (80% acetonitrile, 0.1% formic
acid). The elution gradient was 5–50% B solution in 30 min.

The analytical column was coupled to the MS instrument
using a microflow nebuliser. The equipment was controlled by
HyStar software (Bruker); generated spectra were processed
using DataAnalysis (Bruker) and data base searches were
performed via Mascot MS/MS Ions Search (17). The experi-
ment was performed in separate runs using either positive or
negative ion mode. Ions selected in the positive mode (multiply
charged ions) were run with MS/MS for identification. To
detect tyrosine sulfations, which are more stable in negative ion
mode, the polarity of the instrument was switched (18). To
locate the positions of the sulfation sites the protein was
digested with trypsin to generate peptides which give more
detailed structural information. The peptide map identified
Ucma protein with 10 peptides matching the theoretical
sequence covering amino acids 68–135 of total Ucma, thus rep-
resenting nearly the complete secreted form of Ucma (amino
acids 65–138). The mass shifts corresponding to a sulfate group
in acidic and basic amino acids: the 111 C-terminal amino acids
were found in various other species including rat, dog, clawed frog,
and zebra fish, among others (Fig. 1D). The putative furin cleav-
age site is conserved in most species analyzed. In contrast,
paralogous genes or pseudogenes were not revealed after
screening the published murine genome.

**UCMA, A NOVEL CARTILAGE-SPECIFIC SECRETED PROTEIN**

The deduced amino acid sequence predicts a 27-amino acid
signal peptide as calculated by the web-based signal peptide
prediction program SignalP 3.0 (Fig. 1A). Ucma contains a
RGKR/S consensus sequence predicting processing by a furin-
like protease (see below).

Recently, the same gene has been identified independently in
an EST project as a transcript from a human fetal cartilage
library (8).

Interestingly, the C-terminal fragment is exceptionally rich
in acidic and basic amino acids: the 111 C-terminal amino acids
contain 27 acidic residues and 21 basic residues. Further con-
served functional domains, however, could not be identified by
means of bioinformatic analyses. Murine and human mRNA
and amino acid sequences exhibit a homology of ~65 and 80%,
respectively. Highly conserved orthologues of Ucma can be
found in various other species including rat, dog, clawed frog,
and zebra fish, among others (Fig. 1D). The putative furin cleav-
age site is conserved in most species analyzed. In contrast,
paralogous genes or pseudogenes were not revealed after
screening the published murine genome.

**UCMA IS PROTEOLYTICALLY PROCESSED AND CONTAINS TYROSINE
SULFATES**—For in vitro assays on the physiologic function of
UCMA, and for analysis of potential posttranslational modific-
ations, recombinant (His)6- and FLAG-tagged Ucma protein
was produced in HEK293EBNA cells. In cell lysates the anti-
FLAG antibody detected a major band at about 18 kDa, corre-
sponding to the full-length Ucma protein including His and
FLAG tags (Fig. 2A, Lys) and a minor band migrating at ~13
kDa. Ucma purified from cell culture supernatant showed only
the 13-kDa band, indicating processing of Ucma during or after
secretion (Fig. 2A, SN). Fig. 2B displays the purified Ucma
protein in a Coomassie-stained PAA gel. The N-terminal sequence
of recombinant Ucma (SPKSRDE . . . ) as determined by amino
acid sequencing indicated that Ucma was proteolytically pro-
cessed by a furin-like protease into a C-terminal fragment of 74
amino acid residues (Ucma-C), containing the His-FLAG-Tag
at the carboxyl end, and an N-terminal fragment of 37 amino
acids (Ucma-N) (Fig. 1A) (19). The actual molecular mass of the
secreted C-terminal Ucma fragment (Ucma-C) was determined
to 11510 Da using MALDI-TOF MS, matching the theoretically
predicted molecular weight of its amino acid sequence includ-
ing His and FLAG tags.

Polyclonal rabbit antibodies were raised 1) against a Ucma
peptide containing the SPC cleavage site (TAGL-1) (8), and 2)
against recombinant Ucma-C (UCMA-1). Western blot analy-
sis showed that the TAGL-1 antibodies only detected the
uncleaved form of Ucma, while UCMA-1 antibodies detected
both Ucma-C and the uncleaved Ucma precursor in lysates of
HEK293-EBNA cells transfected with Ucma (Fig. 2C).

The presence of several tyrosine residues in an environment
rich in acidic amino acids raised the question, whether Ucma is
post-translationally modified by tyrosine sulfation which is fre-
quently found in secreted and membrane-associated proteins
(20, 21). To address this question, tyrosine sulfation of the
secreted 13 kDa Ucma-C was analyzed by MALDI-TOF MS. In
two independent preparations protein species were detected
with a molecular mass higher than predicted, indicating sulfu-
ation at 1 or 2 positions (Fig. 3A, arrows). For further localization

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3 U. Dietz et al, manuscript in preparation.
of the sulfation positions, the protein was cleaved with trypsin. Three peptides containing tyrosine residues were further analyzed: 1) “QWHYDGLYPSYLYNR” (amino acids 121–135); 2) “REYYEEQR” (amino acids 87–94); and 3) “EYEEQRNENFVEEQKR” (amino acids 88–105). Peptide 1 contains three potential sites for tyrosine sulfation. Switching polarities for...
this peptide indeed revealed the location of tyrosine sulfates within this peptide. Using a different ionization technique the previous results on peptide 1 were confirmed, but with the additional finding that peptide 1 occurred with 0, 1, 2, or 3 sulfates in relative peak ratios of approximately (1.1:0.1:0.03); thus 0–1 sulfated tyrosines were most common (Fig. 3B). In contrast, peptides 2 and 3 could not be shown to contain tyrosine sulfates.

Ucma Is Predominantly Expressed in Resting Chondrocytes—To determine the expression pattern of Ucma during embryonic and postnatal development, we performed RNA in situ hybridization on paraffin-embedded mouse embryos and organs using two independent antisense RNA probes specific for the coding region or for the 3′-untranslated region. Earliest Ucma signals were detectable at E13.5 at the distal ends of vertebrae (Fig. 4A, panels a and b). At E15.5 Ucma message was observed in the distal and peripheral resting zone of epiphyseal cartilage of the developing limbs (Fig. 4B, panels c and d), and particularly strong in vertebrae (Fig. 4A, panels c–e). The expression in the backbone declined during development, and at post-natal day 60 only sporadic chondrocytes were positive for Ucma mRNA (data not shown).

In the developing tibia first Ucma-positive cells were visible at E15.5 in distal and peripheral zones of the epiphysis (Fig. 4B, panels c and d). Three days later Ucma was expressed in most chondrocytes of the upper (“resting zone”, rz) and peripheral zone of the epiphysis and to some extent in laterally located proliferating chondrocytes (pz), while it was absent from the hypertrophic zone (hz), the central part of the proliferating zone and the joint surface (Fig. 4B, panels g and h). Notably, the strongest expression always resided in the periphery of the epiphyses (Figs. 4B, panels g and h and 5B). A similar distribution in embryonic epiphyseal cartilage was reported recently by Tagariello et al. (8) using radioactive in situ hybridization. Postnatally, Ucma mRNA expression withdrew completely from the center of the epiphyses in the area of the future secondary ossification center (Fig. 4B, panels i–k, arrows) before chondrocytes started to undergo maturation, as shown here for the tibia (Fig. 4B, panels i–l). At P14 Ucma message was restricted to chondrocytes at the periphery of the growth plate (Fig. 4B, panels o and p; Fig. 4, panel j), and at P150 only a few positive cells remained in the corresponding area, predominantly within the proliferating zone (Fig. 4B, panels s and t).

Control hybridizations (Fig. 4, A, panel f and B, panels a, e, i, m, and q) of parallel consecutive sections demonstrate that Ucma expression is confined to chondrocytes expressing collagen type II, but in a much more restricted pattern. The data indicate that Ucma defines a subset of early differentiating chondrocytes in the distal, peripheral zone of the epiphysis expressing already type II collagen, while chondrocytes from the hypertrophic zone expressing collagen type X (Fig. 4B, panels b, f, j, n, and r) did not reveal detectable levels of Ucma mRNA.

Unprocessed Ucma Is Secreted into the Extracellular Cartilage Matrix—To examine the distribution of the Ucma precursor, we generated polyclonal rabbit antibodies against a 17-amino acid peptide of the region overlapping the furin cleavage site in Ucma. This antibody (TAGL-1) recognized the unprocessed form of Ucma (UCMA-C, 13 kDa). In the supernatant (SN) Ucma-C and a partially processed form dominate. B, purified recombinant Ucma-C resolved by SDS-PAGE and stained with Coomassie Blue. C, Western blotting of recombinant Ucma-C and lysate of Ucma-transfected HEK293EBNA cells. The TAGL-1 antibody prepared against a peptide spanning the SPC cleavage site recognizes only the Ucma precursor in the cell lysate, while the UCMA-1 antibody prepared against Ucma-C binds to both Ucma precursor and Ucma-C.

Unprocessed Ucma Is Secreted into the Extracellular Cartilage Matrix—To examine the distribution of the Ucma precursor, we generated polyclonal rabbit antibodies against a 17-amino acid peptide of the region overlapping the furin cleavage site in Ucma. This antibody (TAGL-1) recognized the unprocessed Ucma precursor, but not Ucma-C, in Western blot analyses (Fig. 2C). Immunohistochemical analyses on paraffin sections of E18.5 and newborn mice using the TAGL-1 antibody localized the Ucma precursor to resting cartilage in the distal zones of epiphyses (Figs. 5 and 6), vertebral (supplemental Fig. S1b), sternal (supplemental Fig. S1, e and e'), and rib (supplemental Fig. S1h) cartilage in a pattern very similar to Ucma mRNA (Fig. 5 and supplemental Fig. S1). Strong immunostaining of uncleaved Ucma was found in the extracellular matrix, but could only be visualized after hyaluronidase pretreatment of the sections (Figs. 5, panel e' and 6, panels a' and c', and supplemental Fig. S1e').

This finding is consistent with the extracellular localization of Ucma by immunofluorescence (8). By electronmicroscopical analysis of cartilage using the immunogold labeling technique unprocessed Ucma was found associated with collagen fibrils in the extracellular matrix (Fig. 7A), but also in pericellular lacunae (Fig. 7, B and B'), and, as expected, in the RER (Fig. 7, B and B').

The C-terminal Fragment of Ucma Is Persistent in the Cartilage Matrix—In contrast to the unprocessed form of Ucma, Ucma-C as detected by the UCMA-1 antibody was found in all cartilage areas, including proliferating and hypertrophic zones (Figs. 5 and 6). In the fetal epiphysis, Ucma-C was also seen in the upper surface zone which is negative for Ucma mRNA.
expression and Ucma precursor (Fig. 5, panels d–f). This may be a result of enhanced diffusion of the small C-terminal fragment within the cartilage matrix. A strong immunoreaction for Ucma-C was also seen in the proliferating and upper hypertrophic zone, both in the fetal and post-natal growth plate (Figs. 5, panel l and 6, panels b, d, f, and supplemental Fig. S1, panels c and f’), where the antibody TAGL-1 for unprocessed Ucma was negative. This indicates complete processing of Ucma in these zones and persistence of Ucma-C in the cartilage matrix at the site of initial deposition for several days or even weeks after secretion and processing, while chondrocytes continue to differentiate and mature to hypertrophy (see also “Discussion”).

In Vitro, Ucma Is Expressed during Early Stages of Chondrocyte Differentiation—To investigate Ucma expression during chondrocyte differentiation in vitro, a variety of murine mesenchymal cell lines and primary skeletal cells were tested for Ucma expression by RT-PCR. Among cell lines only chondrogenic ATDC5 and MC615 cells revealed substantial Ucma expression, whereas Ucma expression was absent from the mesenchymal progenitor cell lines C3H10T1/2 and C2C12. Only minor levels of Ucma mRNA were detectable in MC3T3-E1 pre-osteoblasts and NIH3T3 fibroblasts (Fig. 8A). RT-PCR analysis furthermore revealed high levels of Ucma mRNA expression in primary murine rib chondrocytes (RC) as compared with primary osteoblasts from murine calvariae (CC) (Fig. 8C). These analyses confirmed the predominant expression of Ucma in resting chondrocytes observed by in situ hybridization (Fig. 4).

Changes in Ucma expression during chondrocyte differentiation were investigated using a subclone (4C6)4 of MC615 chondrocytes (9). Clone 4C6 exhibited a strong induction of type II collagen (Col II) expression after 1 week in culture, reaching nearly the same level as that of primary rib chondrocytes. In later stages (2 to 5 weeks after seed-

4 C. Surmann-Schmitt, N. Widmann, K. von der Mark, and M. Stock, manuscript in preparation.
agreement with a similar study on the time course of \textit{Ucma} expression in differentiating ATDC5 cells (8) and indicate that \textit{Ucma} expression in chondrocytes peaks in an early maturation stage following expression of \textit{Col II}, while it declines with the onset of \textit{Col X} expression.

**BMP-2 Represses Ucma Transcription in Chondrogenic Cells**—
The finding that \textit{Ucma} expression started in early chondrocytes in the distal zones of epiphyseal cartilage after type II collagen, but disappeared with chondrocyte maturation and hypertrophy raised the question, how \textit{Ucma} expression may be regulated. Tagariello \textit{et al.} (8) have shown that \textit{Ucma} expression in the developing limb is \textit{Indian hedgehog} independent. To investigate a potential regulatory effect on \textit{Ucma} expression by BMP factors, which have been shown to promote chondrocyte maturation toward the hypertrophic phenotype \textit{in vitro} and \textit{in vivo} we treated chondrogenic MC615 cells and primary murine rib chondrocytes with increasing amounts of BMP-2 for 24 h (22). Northern blot analysis revealed a dose-dependent repression of \textit{Ucma} mRNA expression in response to BMP-2 in both MC615 cells and primary chondrocytes (Fig. 10A). While down-regulating \textit{Ucma} expression, BMP-2 enhanced the expression of the hypertrophic chondrocyte markers \textit{Col X} and \textit{Runx2} in MC615 cells as shown by real-time RT-PCR (Fig. 10B). BMP-dependent down-regulation of \textit{Ucma} expression could also be confirmed at protein level using cell lysates of primary rib chondrocytes stimulated with 100 ng/ml BMP-2 in a Western blot analysis (Fig. 10C). These results implicate that BMP-2 may have a role in the suppression of \textit{Ucma} during chondrocyte maturation and hypertrophy.
Ucma-C Suppresses Osteogenic, but Not Chondrogenic, Differentiation—For the analysis of possible Ucma-dependent effects on cell growth and differentiation, chondrocytes and a number of chondrocytic and osteoblastic cell lines were treated with purified recombinant Ucma-C protein in vitro. After treatment of primary murine rib chondrocytes or C3H10T1/2 cells with Ucma-C doses ranging from 125 ng/ml to 1000 ng/ml for up to 11 days, no significant alterations on cell growth were observed (data not shown). Also, treatment of MC615 cells or primary murine chondrocytes with variable amounts of purified recombinant Ucma-C did not significantly change expression of chondrogenesis-related genes as determined in a gene expression array (GE-Array Mouse Osteogenesis Microarray, Superarray) and by real-time RT-PCR (data not shown).

Interestingly however, Ucma impaired osteogenesis in vitro. After treatment of primary murine rib chondrocytes or C3H10T1/2 cells with Ucma-C doses ranging from 125 ng/ml to 1000 ng/ml for up to 11 days, no significant alterations on cell growth were observed (data not shown). Also, treatment of MC615 cells or primary murine chondrocytes with variable amounts of purified recombinant Ucma-C did not significantly change expression of chondrogenesis-related genes as determined in a gene expression array (GE-Array Mouse Osteogenesis Microarray, Superarray) and by real-time RT-PCR (data not shown).

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were also significantly reduced by Ucma-C in a dose-dependent manner after 7 days (Fig. 11, B and C). Impairment of osteogenic differentiation by Ucma-C was confirmed in primary mouse calvarial cells (Fig. 11, E and F). Ucma-C reduced expression levels of bone specific marker genes osteocalcin and bone sialoprotein (Bsp) as determined by real-time RT-PCR (Fig. 11E) and RT-PCR (Fig. 11F). Ucma-dependent effects on osteogenic differentiation were also analyzed using hMSCs that were subjected to osteogenic differentiation. Differentiated hMSCs treated with Ucma-C revealed reduced mRNA levels of osteoblast markers osteocalcin (OCN), and collagen type I (COL1A1), furthermore supporting an antagonistic role of Ucma in osteogenesis (supplemental Fig. S2). Consistent with this notion, expression of endogenous Ucma mRNA in MC3T3-E1 cells was down-regulated during β-GP- and AA-induced differentiation, (Fig. 11D).

These results point to an antagonistic effect of the chondrocyte-derived secreted factor Ucma on osteogenesis and suggests an inhibitory role for Ucma-C in the cross-talk between chondrogenesis and osteogenesis in skeletal development.

DISCUSSION

In this work we present a novel type of a small, secreted cartilage-specific protein, which is processed extracellularly in at least two peptides. Both in situ hybridization and immunohistochemistry show that in situ Ucma is expressed only in cartilage. This is consistent with results from an analysis of Ucma expression in mouse tissues by Northern hybridization (8). According to its major site of expression we termed this novel protein “unique cartilage matrix-associated protein” (Ucma). It is highly conserved among numerous vertebrate species including mammals, amphibians, and fish. In birds (chicken), however, orthologues could not be identified by bioinformatic analyses. So far it is not clear, whether this is due to incompleteness of the chicken genome sequence available in databases, or actually reflects the absence of Ucma homologues in chicken. Especially in an evolutionary context it would be interesting if birds actually lacked this gene.

The Ucma gene codes for a small, secreted protein with a conserved cleavage site for subtilisin-like pro-protein convertases (SPCs) such as furin. Recombinant murine Ucma protein purified from culture supernatant of transfected HEK293 EBNA cells consisted only of the 74-amino acid C-terminal fragment of Ucma (Ucma-C). N-terminal sequencing of Ucma-C confirmed cleavage of the Ucma precursor protein by a SPC at the predicted RGKR-S consensus site. SPCs convert a number of precursors of secreted proteins including members of the TGFβ superfamily and most neural and peptide hormones (23). In cartilage a number of protein precursors are known to be processed by SPCs including BMP-like factors, collagen α1(XI), and chondromodulin-1 (23–26). Cleavage may occur intracellularly during secretion after removal of the signal peptide as shown for GDF5 in Xenopus embryogenesis and during mouse limb formation (26). Yet, proprotein cleavage can also occur at the cell surface or within the extracellular compartment (23). For example, this latter mode of cleavage has been demonstrated for the activation of the BMP antagonist nodal during mammalian antero-posterior axis formation in early embryogenesis (27). The fact that the uncleaved Ucma precursor protein was located in the extracellular cartilage matrix indicates that Ucma processing takes place extracellularly or at the cell surface.

Interestingly, the two SPC family members PACE4 (SPC4) and PC6A were detected attached to heparin residues of heparan sulfate proteoglycans in the extracellular matrix of HEK293 cells (28). This may be of relevance since heparan sulfate containing proteoglycans are abundant in cartilage matrix and thus may represent a source of SPCs responsible for cleavage of Ucma in vivo (29). Another candidate for Ucma processing in HEK293EBNA cells and in cartilage is furin, the prototype of this class of proteases, which is present in virtually every mammalian cell at different levels (30, 31). Different SPCs have been reported to be expressed in cartilage including furin, PACE4 (SPC4), SP6C, and SP7 (26, 31, 32). It will be interesting to identify the SPC that is responsible for Ucma cleavage under physiologic conditions.

The high conservation of the amino acid sequence of Ucma-C and our finding that this fragment affects osteoblast differentiation indicates that the C-terminal part may reflect the biologically active form of Ucma. While the N-terminal peptide (amino acids 28–64) exhibits a homology of 75% between mouse and human, the homology of the C-terminal peptides of both species are conserved to 89%. Comparing the amino acid sequences of six vertebrate species, the C-terminal peptide is completely conserved at 42 of 74 amino acid residues (57%).

Recombinant Ucma secreted from HEK293EBNA cells was found to be sulfated at up to two tyrosine residues. In skeletal...
tissues many proteins, especially leucine-rich repeat proteoglycans of the extracellular matrix like fibromodulin, lumican, and osteoadherin undergo tyrosine sulfation (18). Assuming tyrosine sulfation of Ucma protein also in cartilage in vivo, this similarity to many extracellular proteoglycans may point to a role for Ucma as a component of the extracellular cartilage matrix. Generally, tyrosine sulfation is implicated in protein-protein interaction, enhancing affinities of the binding partners, and thereby also increasing receptor-ligand interactions of a number of signaling molecules and their receptors (33–35). Currently, it is still open whether Ucma exerts its physiologic role as a component of the extracellular matrix or rather as a paracrine signaling molecule.

Ucma expression largely coincides with Col2a1 expression but declines with onset of chondrocyte hypertrophy as measured by Col101 expression both in situ and during chondrogenic differentiation in vitro as shown here in MC615/4C6 cells and in differentiating ATDC5 cells (8). This indicates that Ucma is connected to the hyaline phenotype of resting and proliferating chondrocytes. The fact that we identified Ucma as one of the major down-regulated genes in retinoic acid-treated dedifferentiated chondrocytes is consistent with this notion.

How Ucma expression is regulated during chondrocyte differentiation remains to be elucidated. Ihh signaling does not seem to control Ucma expression in embryonic limb development (8). In this work, however, we show that BMP-2 is certainly a good candidate for a negative regulator of Ucma with onset of chondrocyte maturation.

The expression pattern of Ucma mRNA in distal zones of epiphyseal and vertebral cartilage largely coincided with the distribution of the unprocessed Ucma precursor, as revealed with the antibody TAGL-1 specific for uncleaved Ucma. In contrast, UCMA-1 antibody detecting both Ucma-C and the uncleaved precursor revealed a much wider immunostaining pattern in all zones of cartilage. It is well possible that the small 74-amino acid residues Ucma-C diffuses readily though the cartilage matrix, but the strong pericellular reaction of Ucma-C around proliferating and hypertrophic chondrocytes may be better explained by a high protein stability or stability of the antigenic epitope of Ucma-C. This hypothesis is supported by the strong immunostaining reaction with the UCMA-1 antibody in growth plates of tibiae from adult mice, where TAGL-1-dependent staining for the uncleaved Ucma precursor was barely detectable.

Recombinant Ucma-C has no measurable effect on proliferation, differentiation or matrix synthesis of primary chondro-
cytes or chondrocyte lines. However, the expression of osteogenic markers in MC3T3-E1 cells that had been induced to differentiate to osteoblasts was significantly inhibited by Ucma-C, suggesting a paracrine action of chondrocyte-derived Ucma on bone cell development. Similar findings in primary mouse calvarial cells and human menesenchymal stem cells support this hypothesis.

There is numerous evidence for mutual interactions between chondrocytes and bone cells in the regulation of chondrogenic versus osteogenic differentiation (36, 37). Thus, Ucma may represent a paracrine factor supporting the balance between chondrogenic versus osteogenic differentiation at the cartilage interface with perichondrium or periosteum in the developing skeleton. So far, reports on paracrine factors mediating signals between different cell types involved in bone and cartilage development focused mainly on the Ihh/PTHrP system, and the control of vascularization by VEGF produced by hypertrophic chondrocytes. Several other paracrine factors including FGF18 derived from the perichondrium have been reported to negatively influence osteoblast differentiation (2, 7). In contrast, Ucma may act as a direct link between epiphyseal chondrocytes and pre-osteoblasts in the control of a well concerted and balanced formation of cartilage and bone.

The mechanism of such regulatory functions is, however, still unclear. Ucma-specific cellular receptors have not been identified, yet. Because of its highly polar character and the potential for multiple protein interactions, matrix-bound Ucma-C may exert indirect effects by binding growth factors or their inhibitors and controlling their release.

The same features, however, propagate Ucma-C also as an integrative constituent of the cartilage matrix, where it may control the assembly of collagens, proteoglycans and glycoproteins. The generation of Ucma-deficient and Ucma-overexpressing mice is in progress to answer these questions.

Acknowledgments—We thank Britta Schlund and Eva Bauer for excellent technical assistance.

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