Chondromodulin-I (ChM-I) is a small glycoprotein that is abundant in fetal cartilage. Mature chondromodulin-I is processed from a larger precursor form, presumably at a proteolytic site RERR-ELVR. The precursor, mature chondromodulin-I and two processed products, the remnant left after removal of mature chondromodulin-I and a smaller, unglycosylated form, were identified using antipeptide antisera. The products of chondromodulin-I precursor processing were seen in cultured chondrocytes, a stable long-term culture chondrosarcoma cell line, as well as Chinese hamster ovary (CHO) cells transfected with an expression plasmid that contained cDNA coding for the chondromodulin-I precursor. Pulse-chase analysis allowed a processing pathway to be analyzed for chondromodulin-I. To further dissect the processing events, three constructs that express recombinant wild-type or mutant chondromodulin-I were transfected into CHO cells. We showed that chondromodulin-I is cleaved intracellularly at the predicted cleavage site, and that the mature glycopeptide is rapidly secreted immediately after processing. The chondromodulin-I precursor has a short half-life and is not readily apparent in tissue samples, suggesting that chondromodulin is not a member of the juxtacrine family of growth factors, despite some similarities. The smaller unglycosylated form of chondromodulin-I was only observed in cartilage and not in short-term cultures or transfected cells, suggesting an extracellular processing event. No processing occurred when the precursor cleavage site was mutated to RERQ-ELVR or when precursor chondromodulin-I was expressed in the furin-deficient CHO cell line, suggesting the involvement of furin in processing.

Post-translational Processing of Bovine Chondromodulin-I*

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Chondromodulin-I (ChM-I)† is an abundant glycoprotein in fetal cartilage (1) that is largely restricted to cartilage on the basis of Northern blot analysis (2). The pattern of ChM-I expression and the fact that purified ChM-I inhibited DNA synthesis and proliferation in vascular endothelial cells as well as tube morphogenesis in vitro (3) suggest that one role for ChM-I is as an inhibitor of angiogenesis. However, the abundance of ChM-I in fetal cartilage suggests that it may have other roles in chondrocyte growth modulation. Regulatory factors involved in chondrocyte differentiation and cartilage metabolism have recently been reviewed (4, 5).

Early in chick development ChM-I mRNA can also be found in the heart and retina (6). In the mouse, ChM-I mRNA is found in the thymus and eye but at a lower level than in cartilage (7). The mRNA for ChM-I was shown to be abundant in fetal cartilage and, although present in adult cartilage, was reduced to 5% of the fetal level (8). Immunohistochemistry and in situ hybridization localized ChM-I and its mRNA to the interterritorial matrix of the avascular zones of growth plate cartilage (3). ChM-I stimulates DNA synthesis and proteoglycan biosynthesis in the presence of basic fibroblast growth factor 2 (2).

Mature ChM-I is a 121-amino acid glycoprotein that is derived from the C terminus of a 335-amino acid precursor (2). The N terminus, as isolated from cartilage, is ELVR (1, 2), and this glycosylated protein is referred to in the present study as 28-kDa ChM-I. Little is known about the post-translational processing of the ChM-I precursor. The precursor remnant ChM-I presumably represents the product that results from cleavage of ChM-I and would have a C-terminal sequence of RERR. This presumed proteolytic site, which matches the consensus sequence for furin-pro tease cleavage, is conserved between species (9). It is not clear whether ChM-I is processed intra- or extracellularly. The precursor remnant may be degraded intracellularly, may remain on the cell surface, or may be rapidly internalized and degraded. A smaller, unglycosylated ChM-I form of 82 amino acids with a N-terminal DAEP, which is abundant in fetal cartilage (1), derives from the C terminus of 28-kDa ChM-I and is referred to here as 9-kDa ChM-I.

The overall structure of ChM-I is analogous to the family of juxtacrine growth factors that are derived from membrane-anchored precursors (10). Members of this family include transforming growth factor-β, tumor necrosis factor α, heparin binding epidermal growth factor, and amphiregulin. The precursor and soluble growth factor forms have functions that are separate from each other. For example, the precursor form of transforming growth factor-β is a juxtacrine growth factor that mediates signaling, proliferation, and adhesion of the neighboring cells (11). The processed, secreted soluble form of transforming growth factor-β can diffuse freely and is a potent paracrine and autocrine growth factor.

To analyze the processing pathway for ChM-I and to determine whether it might be a candidate member of the juxtacrine family of growth factors, we generated antipeptide antisera that detect and distinguish between processed and precursor ChM-I forms. We show for the first time that the ChM-I precursor is processed intracellularly. Transfection of mammalian cell culture systems with ChM-I constructs and pulse-chase analysis to establish a precursor-product relationship revealed...
a processing pathway similar to that seen in cultured chondrocytes. Our results support the hypothesis that furin is the protease responsible for post-translational processing of ChM-I.

**Materials and Methods**

All tissue culture supplies were obtained from Life Technologies, Inc. or Fisher. All chemicals were purchased from Sigma or Roche Molecular Biochemicals. [35S]Cysteine-methionine was obtained from NEN Life Science Products. Restriction enzymes and molecular biology reagents were obtained from U. S. Biochemical Corp., Stratagene, Roche Molecular Biochemicals, or Life Technologies. Plasmids pCR2.1TA and pCDNA3.1/Zeo(+) and frozen competent cells were obtained from Invitrogen (San Diego, CA). ChM-I was isolated from cartilage with 4 M guanidine-HCl as previously described (1).

**Cell Culture, Transfection, Treatment, and Metabolic Labeling**

**Cell Culture**—Bovine articular cartilage from the metacarpal phalangeal joint or rib growth plate cartilage were obtained from fetal animals. Chondrocytes were isolated by digestion for 2 h with 2 mg/ml Pronase in Dulbecco’s modified Eagle’s medium (DMEM) with gentle shaking (90 rpm), followed by an 18-h digestion with 0.5 mg/ml collagenase with gentle shaking (50 rpm). Freshly isolated cells were either lysed directly in sample buffer for Western blot analysis or plated in monolayer culture for metabolic labeling and subsequent immunoprecipitation.

Chondrocytes and Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (50 μg/ml streptomycin, 50 units/ml penicillin, and 10 μg/ml gentamicin). A mutant CHO cell line deficient in furin (RPE40; Ref. 12) was grown in Ham’s F-12 medium supplemented with 10% fetal bovine serum. The same antibiotics and a generous gift from Dr. Thomas Moehring.

Long-term culture chondrosarcoma (LTC) cells were a generous gift from Dr. Kurt Doege and Dr. James Kimura and were passaged from Thomas Moehring. The cell line established by Kucharska et al. (13) in Ham’s F-12 medium supplemented with antibiotics and 10% fetal calf serum was a generous gift from Dr. Kurt Doege and Dr. James Kimura and was passaged from Thomas Moehring.

**Transfection**—CHO cells were plated at 2.5 × 10^5 cells per well in a six-well plate (35-mm well diameter). The following day, cells were transfected for 5 h with 1 μg of plasmid that had been mixed and incubated with 6 μl of LipofectAMINE (Life Technologies) for 30 min under serum-free conditions. Serum was then added to a final concentration of 10%, and incubation continued for up to 48 h. A similar protocol was used for transfection of RPE40 cells (12).

**Antibodies**—Antibodies were purified by either Western immunoblotting or immunoprecipitation. For selection of CHO transfectants that were stably expressing ChM-I, cells were diluted 1:10 48 h after transfection and incubated in DMEM containing 1 mg/ml zeocin (Invitrogen) until zeocin-resistant cells formed visible colonies. Colonies that were zeocin resistant were trypsinized within cloning rings, transferred to 24-well plates, and selected for clonal purity by limiting dilution.

**Metabolic Labeling and Cell Lysis**—For metabolic labeling, CHO transfectants or chondrocytes in monolayer culture were plated in 100-mm tissue culture dishes and labeled for 4 h with 250 μCi/plate [35S]Cysteine-methionine in cysteine- and methionine-free DMEM containing 10% dialyzed fetal calf serum. The cell medium was removed and saved for further analysis. Cells were rinsed twice with phosphate-buffered saline and lysed by incubation (and gentle shaking) in 1 ml of lysis buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5% deoxycholate, 1% Nonidet P-40, and protease inhibitors: 50 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) at 4 °C for 30 min. Cells were then scraped and sonicated or passed through a 20-gauge needle for further lysis. For pulse-chase analysis, after the indicated pulse time, cells in monolayer were rinsed and incubated in complete DMEM for the indicated chase time.

**ChM-I Plasmid Constructs**

pChM-I and ChM-I Plasmid Constructs—RNA from bovine epiphyses was isolated as previously described (8) and used as the template for reverse transcription-PCR using primers 6U23 (CAGTCATGCTGTTCTGGGTT) and 2164L22 (CTGACAGGAGGATCAAGCTGTC) and 1111L26 (TGGCTATGCTCCTTTAGCTCACG). The product was cloned into pCR2.1 (Invitrogen) and then subcloned into pCDNA3.1/Zeo at the BamHI and NotI sites.

**Mutagenesis**—A PCR product corresponding to full-length precursor ChM-I was prepared with primers 786U19 (CGTGTACGTGGGAGGTTCT) and 2164L22 (CTGACAGGAGGATCAAGCTGTC) (see Fig. 4A). These primers are in the pCDNA3.1 vector, outside the CDNA-derived cassette. Modified half-length precursor ChM-I DNA fragments were generated with overlapping extensions. Owing by PCR with these primers generate a full-length PCR product containing the desired mutations of R214S and E215Q (pPreChM-I; primers 1576SQU30, ATCCAGAGGAGAAAGGACACACTGTTAGAA; and 1584SCL30, TCTTCTTCGTTGACATTCTTTTATGCA). The product was then used to replace the wild-type ChM-I in plasmid ChM-I. Clones were then sequenced to further verify incorporation of the correct sequence (DNA Sequencing Core Laboratory, University of Florida, Gainesville, FL).

**Analyses of ChM-I Products by Western Blotting and Immunoprecipitation**

**Generation of ChM-I-specific Antipeptide Antiserum—**Two peptides, CWKPYTPKIEQRR (USF-PN2) and DPYHQQEGESMTFDPLRC (USF-PN3), were synthesized, coupled to keyhole limpet hemo- cyanin, and conjugated to their terminal cysteine residues for use to immunize rabbits to generate antisera AN71 and AN73, respectively (Anaspec Inc., San Jose, CA, Fig. 1). All antisera were positive for the immunizing peptides in an enzyme-linked immunosorbent assay. Crude antisera were affinity purified further by adsorption of antibodies to peptides that had been immobilized to Sepharose (SufoLink, Pierce). Purified antibodies were eluted with Immunopure Gent Ab elution buffer (Pierce). The specificity of the AN73 antisera to chondromodulin was analyzed by passing it down affinity columns containing either immobilized USF-PN2 or USF-PN3 and analyzing both the void (unbound) fractions and eluted fractions in Western blots.

**Western Blot Analysis—**Samples resuspended in sample buffer were separated by SDS-polyacrylamide gel electrophoresis (PAGE; NuPAGE 4–12% Bis-Tris (2-bis(2-hydroxyethyl)aminomethyl)-propane-1,3-diol) gel; Novex, San Diego, CA) under reducing conditions, and transferred by wet transfer to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Affinity-purified antisera AN71 and AN73 were used at dilutions of 1:5000 overnight. Antibodies were visualized with a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Tropix Inc., Bedford, MA) and developed with a chemiluminescent substrate, CSPD™, using the Western-Light chemiluminescent detection system (Tropix).

**Immunoprecipitation—**The cell lysate and medium (1 ml) were incubated with antisera AN71 or AN73 or the corresponding preimmune sera (5 μl) and a protein A-agarose suspension (Life Technologies; 30 μl) for 3 h at 4 °C and washed extensively with cell lysis buffer. The resulting samples were resuspended in sample buffer and separated by SDS-PAGE under reducing conditions. The gel was treated with Enhansify solution (NEN Life Science Products), and bands were detected with Biomax film (Eastman Kodak Co.) or a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for densitometric quantitation.

**Results**

**Generation of Anti-ChM-I Antipeptide Antiserum—**Two forms of ChM-I have been detected in fetal bovine cartilage: a glycosylated form with the N-terminal sequence ELVR (28-kDa ChM-I) and a smaller unglycosylated form with an N-terminal sequence of DAEP (9-kDa ChM-I; Ref. 1). The N-terminal two-thirds of ChM-I, remnant ChM-I, may remain on the cell surface after 28-kDa ChM-I processing from the precursor, may be internalized, or may never appear on the cell surfaces and be degraded intracellularly. 9-kDa ChM-I is preceded by the sequence SVQE, which does not conform to the furin cleavage consensus sequence.
Post-translational Processing of Bovine Chondromodulin-I

Two anti-peptide antisera, AN71 and AN73, were generated to enable us to target specific domains of ChM-I (Fig. 1). AN71 is generated against the synthetic peptide USF-PN2 (Fig. 1), which contains the neoepitope that would derive from removal of ChM-I from the precursor. AN71 would also detect the residues in USF-PN2 that do not constitute a neoepitope and therefore would be expected to also detect the precursor for ChM-I. AN73 is generated against USF-PN3 (Fig. 1), which is located near the N terminus of 9-kDa ChM-I, and detects the 28- and 9-kDa as well as the precursor ChM-I (Fig. 1).

To visualize the products of ChM-I precursor processing, we analyzed fetal cartilage extracts and isolated chondrocytes by Western blotting, using affinity-purified anti ChM-I antiserum AN73. The 28- and 9-kDa forms of ChM-I could be visualized in Western blots of fetal cartilage tissue extracts but not from freshly isolated chondrocytes (Fig. 2). The glycosylated 28-kDa ChM-I appeared as a broad diffuse band, and the unglycosylated 9-kDa ChM-I was detected as a sharp band. A 7-kDa form of ChM-I could be detected in chondrocytes and at a reduced level in cartilage tissue extract. This 7-kDa form of ChM-I has not been detected previously.

To validate the interaction of AN73 with the 7-kDa band (as well as with 28- and 9-kDa ChM-I), Western analysis was performed with antiserum that had been adsorbed to columns of either Sepharose-immobilized USF-PN2 or USF-PN3. AN73 that had been passed down a column of immobilized USF-PN3 did not show the bands seen in Fig. 2, whereas AN73 that had been passed down a column of immobilized USF-PN2 did show these bands. This indicated that the immobilized immobilizing peptide would efficiently remove immunoreactivity. Conversely, eluate from immobilized USF-PN2 did not react with these bands, whereas the eluate from immobilized USF-PN3 did react (results not shown). Thus, the bands detected by AN73 correspond to proteins containing the USF-PN3 sequence.
AN73. Secreted 28-kDa ChM-I was recovered from culture media (Fig. 3C). A band at ~23 kDa was immunoprecipitated with AN71. This band (p23) is smaller than the ChM-I remnant detected in chondrocytes using the same method and seems likely to be a cross-reacting protein (Figs. 3C, 5, and 6), possibly another protein with an RERR furin cleavage consensus sequence.

Dissecting ChM-I-processing Events in CHO Cells Transfected with Modified pChM-I Constructs—ChM-I processing could be analyzed and the antisera could be further validated by using recombinant ChM-I mutants that either cannot be processed or that have been artificially truncated at the processing site. To dissect ChM-I-processing events and to further confirm the specificity of AN71 and AN73, CHO cells were transfected with ChM-I constructs pChM-I (encoding the wild-type ChM-I precursor), pPreChM-I (encoding a mutant ChM-I precursor that cannot be processed), and pRemChM-I (encoding a mutant ChM-I that has been truncated at the processing site; Fig. 4A) were constructed.

CHO cells were transfected with empty vector pCDNA3.1, pChM-I, pPreChM-I, and pRemChM-I. Fig. 4B shows the Western blot analysis of transfected CHO cells. Vector products were detected with AN71 (left panel) and AN73 (right panel). C, immunoprecipitation with AN73 or AN71 of metabolically labeled CHO cells stably transfected with pChM-I (ChM8.1), pPreChM-I (PreChM2.11), or pRemChM-I (RemChM7.1). Immunoprecipitated material was analyzed by SDS-PAGE and autoradiography.
remnant ChM-I. A remnant is also visible in cells transfected with pChM-I. As expected, the remnant ChM-I was absent in pPreChM-I transfectants, because processing had been disabled by the mutation at the processing site.

To further analyze the expression of products of the ChM-I constructs, cell lysates and culture media from CHO cells that were stably expressing wild-type and mutant ChM-I were immunoprecipitated with either AN71 or AN73 antisera (Fig. 4C). The lysate of CHO ChM8.1 that was stably transfected with wild-type pChM-I contains precursor and cell-associated 28-kDa ChM-I when immunoprecipitated and visualized with AN73. 28-kDa ChM-I was recovered in the cell medium with AN73, whereas the remnant ChM-I form was detected with peptide-purified AN71 anti-remnant antiserum. In contrast, CHO mutant PreChM2.11 expressed only the unprocessed precursor ChM-I and not the 28-kDa ChM-I or remnant ChM-I. CHO ChM-I mutant RemChM7.1 expressed remnant ChM-I but not the precursor or 28-kDa ChM-I. AN71 detected both the precursor and the remnant, because the precursor also contains the USF-PN2 sequence.

The ChM-I remnant (a sharp band at 28 kDa) was immunoprecipitated from LTC cells (Fig. 5) stably transfected with pChM-I. Transiently transfected cells accumulated considerably more precursor and remnant than stably transfected cells (results not shown). This is likely because of overloading of translational and post-translational machinery within the transfected cells.

**Pulse-Chase Labeling to Investigate the Precursor-Product Relationship of ChM-I—I—LTC cells were pulse labeled with [35S]cysteine-methionine for 30 min and chased for various times to determine ChM-I the precursor-product relationship. Pulse-chase analysis was also performed to detect other short-lived, intermediate processed ChM-I forms that may be present in the cells. Cell lysates and media were immunoprecipitated with AN73 anti-ChM-I antisera (Fig. 6A). The bands corresponding to precursor and processed ChM-I in nontransfected LTC cells were scanned and quantitated relative to total counts and plotted against time (Fig. 6B). We were unable to detect the ChM-I remnant in LTC cells unless they were overexpressing ChM-I as a result of transfection with pChM-I. Pulse-chase analysis of pChM-I stably transfected LTC cells using AN71 as the immunoprecipitating antiserum resulted in two bands (Fig. 6C), a transient band corresponding to remnant ChM-I and a stable band that did not show a precursor-product relationship and that corresponded to p23.

Precursor ChM-I declined with time, whereas secreted 28-kDa ChM-I increased correspondingly. A peak of cell-associated 28-kDa ChM-I was detected after 1 h of chase but declined after that, further supporting the hypothesis that there is not a pool of the cell-associated precursor and that the precursor is cleaved into 28-kDa ChM-I and a rapidly degraded remnant. No other intermediate forms were detected. In contrast, transfected CHO cells accumulated considerable intracellular 28-kDa ChM-I. In CHO cells, high levels of precursor and 28-kDa ChM-I were still present after 6 h of chase, suggesting that the cellular processing machinery was overloaded (data not shown).

**ChM-I Processing May Involve Furin Protease—Defective**
The goal of this study was to define steps in the biosynthesis and post-translational processing of the precursor to generate free 28-kDa ChM-I (Fig. 1). Using anti-ChM-I- and anti-remnant ChM-I-specific antisera, we were able to detect and identify the four ChM-I forms previously reported, or speculated to be present, in cartilage, as well as 7-kDa ChM-I, a species that has not been observed previously (Figs. 2 and 3).

Detection of precursor, remnant, and 28-kDa ChM-I in cultured chondrocyte lysates and secreted 28-kDa ChM-I in chondrocyte culture medium (Fig. 3, A and B) suggests that the pathway for ChM-I processing depicted in Fig. 1 is likely to be correct. The proteins synthesized by CHO cells transfected with constructs containing modified ChM-I (Fig. 4) further support the processing hypothesis. The product of pPreChM-I mutated at the predicted protease recognition site was deficient in processing, implying that ChM-I processing was indeed at this site and that an intact signal is necessary for protease cleavage. pRemChM-I with a stop codon that deleted 28-kDa ChM-I from the precursor expressed a product that is similar in size and immunoreactivity to the native remnant ChM-I form seen in chondrocytes (Fig. 3) and transfected CHO cells (Fig. 5), further confirming the identity of remnant ChM-I. Before this study, this processing paradigm had been speculative, because these different ChM-I forms had not been identified.

Cleavage of precursor proteins that generate biologically active proteins and peptides can occur intracellularly, at the cell surface or within the extracellular milieu (16). It was not clear before this study whether ChM-I is processed intra- or extracellularly. There are three possible schemes for ChM-I precursor processing that would generate the active mature 28-kDa ChM-I. In the first, the ChM-I precursor would be processed on the cell surface, leaving remnant ChM-I associated with the cell surface. In the second scheme, the ChM-I precursor may be processed intracellularly, and 28-kDa ChM-I would be independently secreted; the precursor remnant could also be transported to the cell surface. Third, the ChM-I precursor could be cleaved intracellularly; the remnant would be rapidly removed (and would not be detected on the cell surface); and 28-kDa ChM-I would be transported to the cell surface. On the basis of the results of the present study, the third scheme appears to be most likely.

As shown in Fig. 3, precursor, remnant, and 28-kDa ChM-I could be immunoprecipitated from the cell lysates, indicating that processing of the precursor takes place intracellularly, before secretion of the mature 28-kDa ChM-I. If processing takes place on the cell surface, as described in the first scheme, 28-kDa ChM-I would only be associated with the cells if it were bound to the cell surface in some way. This was not observed in the present study. Pulse-chase analysis of LTC cells overproducing pChM-I show that the cell-associated 28-kDa ChM-I is rapidly lost from the cell layer, in parallel to the loss of the 28-kDa remnant, suggesting that the two events are nearly contemporaneous. These findings indicate that processing precedes secretion of 28-kDa ChM-I, which supports the hypothesis that processing of the ChM-I precursor takes place intracellularly.

Intracellular processing suggests that ChM-I may be cleaved from its precursor by a specific intracellular protease, immediately after biosynthesis. Presumably, the recognition site is the RERR that immediately precedes the sequence ELVR at the N terminus of bovine 28-kDa ChM-I. Mutation of R-ELVR to S-QLVR at the predicted processing site (coded for by pPreChM-I precursor (2) suggested the presence of at least two other forms of ChM-I, a membrane-associated precursor and the N-terminal two-thirds remnant that remains membrane associated after removal of 28-kDa ChM-I. The precursor and precursor remnant have not previously been detected.

The goal of this study was to define steps in the biosynthesis and post-translational processing of the precursor to generate free 28-kDa ChM-I (Fig. 1). Using anti-ChM-I- and anti-remnant ChM-I-specific antisera, we were able to detect and identify the four ChM-I forms previously reported, or speculated to be present, in cartilage, as well as 7-kDa ChM-I, a species that has not been observed previously (Figs. 2 and 3).
and PC7/SPC7/PC8/LPC, are responsible for the conversion of precursor proteins into their biologically active forms (16). Precursors are usually cleaved at the general motif (K/R)-(X)n-(K/R), where n = 0, 2, 4, or 6, and X is any amino acid but usually not cysteine (16).

A mutant strain of the CHO-K1 cells, RPE.40, was shown to be resistant to Pseudomonas exotoxin A because of impaired proteolytic processing of the proprotein form that generates the active toxin (17). It was later verified that RPE.40 had a mutated fur gene, which abolished expression of furin protease responsible for Pseudomonas exotoxin A cleavage in the cell line (12). We show in this study that although precursor ChM-I was correctly processed in transfected CHO cells, it was not cleaved in similarly transfected RPE.40 cells (Fig. 7). These data strongly suggest that furin is the protease involved in the cleavage of precursor ChM-I into mature 28-kDa ChM-I at its specific recognition sequence.

The smaller 9-kDa ChM-I found in developing articular cartilage (Fig. 2; Ref. 1) was not detected in freshly isolated chondrocytes, chondrocyte monolayer cultures, or CHO cells that were stably expressing ChM-I. This suggests that it may not be processed directly from the precursor ChM-I but is likely cleaved from secreted 28-kDa ChM-I by a matrix-associated protease, possibly one of the matrix metalloproteinase family.

The P7 form of ChM-I appears to be the product of action by an intracellular protease, because P7 could be detected only in freshly isolated chondrocytes. The sharp band on SDS-PAGE suggests that P7 does not contain the carbohydrate found on 28-kDa ChM-I. It contains the USF-PN3 epitope and does not have a high enough molecular weight to include both this epitope and the C-terminal end of ChM-I. Therefore, it is probably an internal peptide fragment.

No other intermediate forms other than the precursor, remnant, and 28-kDa ChM-I were detected through pulse-chase analysis, suggesting that the 28-kDa ChM-I is directly processed from the precursor ChM-I form. As the immunoprecipitated ChM-I precursor declined with time, the bands for the processed 28-kDa ChM-I (cell associated and secreted) were associated with the cell-associated 28-kDa ChM-I, which is then secreted into the medium. We establish in this study that the different forms of ChM-I, precursor, remnant, and 28- and 9-kDa ChM-I, are present in cartilage. We showed that precursor ChM-I is processed intracellularly, most likely by furin protease, to generate 28-kDa ChM-I, which is then secreted outside the cell. To date, the role of 28-kDa ChM-I as a cartilage-specific antiangiogenic and growth-modulating factor has been elucidated (18, 19). The precursor and remnant have half-lives that are relatively short, suggesting that they do not have a function. It is possible that the extracellular cleavage of 9-kDa ChM-I from 28-kDa ChM-I may enable the smaller, unglycosylated protein to diffuse more rapidly through the matrix, thus modulating the activity of ChM-I. In this context, it is interesting that a relative of ChM-1 has been detected recently in tendon (20). This protein appears to remain in the cell membrane (21, 22) and therefore, unlike ChM-I, may be a candidate juxtacrine factor.