Integrity of cartilage fails in joint disease. The current work aimed to identify candidate active proteinases in joint diseases using an in vitro model for cartilage degradation induced by interleukin-1. A critical event in the process of cartilage destruction in joint disease is the failure of the collagen fiber network to maintain integrity. Proteins binding to the surface of the fibers are likely early points of failure. Fibromodulin, a member of the leucine-rich repeat protein family, is one predominant protein in cartilage and is known for its roles in the formation of collagen fibrils and sustained interaction with these formed fibers. Cleavage removes the tyrosine sulfate-rich region in the N terminus of fibromodulin. Whereas fibromodulin bound to collagen in tissue was digested, purified fibromodulin was not cleaved. In contrast an N-terminal 10-kDa fragment, Gln19-Lys98, of the protein generated by Lys-C digestion contains the cleavage site and was a substrate cleaved by the enzyme in medium from stimulated cultures. In solution, digestion of this substrate with matrix metalloproteinase (MMP)-2, -9, -8, and -13 demonstrated that only MMP-13 was capable to efficiently cleave it. The cleavage product obtained after MMP-13 digestion was identical to that observed in cleaved fibromodulin from cartilage explant cultures stimulated with interleukin-1. MMP-13 treatment of fresh articular cartilage also produced the fragment under study. The elucidation of the enzyme responsible for such cleavage may lead to treatment modalities involving its selective inhibition for patients suffering from arthritis. The known structure of the fragments permits the generation of neo-epitope antibodies to the cleavage site, which can be used to detect ongoing cartilage degradation in patients with arthritic disease, an important adjunct in monitoring disease progression, active disease, and efficacy of treatment.

Fibromodulin is a member of the small leucine-rich repeat protein family. It was originally described as a 59-kDa collagen-binding protein (1). The closest sequence relatives of fibromodulin (2) are lumican (3, 4), keratocan (5), PRELP (proline/arginine-rich end leucine-rich repeat protein) (6), and osteoadherin (7). Fibromodulin is tyrosine sulfated in its N-terminal extension (8). This substitution is suggested to occur in tyrosines located next to acidic residues, although later work has shown a new consensus for the modification of tyrosine residues (9). Two disulfide loops are present in fibromodulin. The most N-terminal loop may have a role in the coordination of metal binding as has been described for two of its relatives, decorin and biglycan (10, 11). Fibromodulin can be substituted on asparagine residues with keratan sulfate chains at four potential sites, within the leucine-rich-repeat region (2, 12).

Binding of fibromodulin to both type I and type II collagen in vitro has been demonstrated and is thought to have an important role in correct fiber formation (1). This binding appears to be independent of disulfide bridging and appears to include a region in the C terminus of fibromodulin (13). Both fibromodulin and lumican have been shown to bind to the same site on the collagen fibril, whereas decorin appears to bind to a separate site (14, 15). Fibromodulin appears also to modify the properties of the completed collagen fiber and is found localized at the gap region with a surface density differing with dimensions of the fiber (1). Fibromodulin null mice have recently been demonstrated to form abnormally thick type I collagen fibrils in tendon (16). They show an increased incidence of arthritis in later life possibly as a result of malfunctioning ligaments (17). In contrast, decorin null mice form abnormally large diameter fibrils in a markedly weakened skin (18). Double knockouts of lumican/fibromodulin show a propensity to joint laxity likely caused by weak tendons (19).

Matrix metalloproteinases (MMPs) appear to have roles in the degradation of the cartilage matrix in osteoarthritis (20, 21). MMP-13 cleaves native collagen and is thought to initiate degradation of the collagen fiber in arthritic conditions (20, 22, 23). While work on the role of fibromodulin in development is increasingly abundant, no studies have attempted to characterize the breakdown of fibromodulin seen in either rheumatoid and osteoarthritic cartilage (24, 25) or explant culture with interleukin-1α (IL-1) (26). In this report we have used a well characterized destructive model of bovine cartilage to elucidate the exact site of cleavage of fibromodulin by an as of yet unidentified protease. The characterization of this site will allow...
the generation of neo-epitope antibodies that could be used as markers of cartilage degeneration in disease.

Neo-epitope antibodies have been used to study cartilage breakdown, more specifically of aggrecan (27, 28) and type II collagen (23, 29). The cleavage of fibromodulin may precede major destruction of type II collagen and may be permissive for this process. This degradation is thought to be a crucial point in joint destruction past which expectation for self-repair of cartilage fails is low. Also, the identification of the enzyme responsible for this degradation of this major cleavage product could lead to treatments aimed at blocking its selective inhibition in patients suffering from arthritic disease. The capacity for early detection and inhibition of this process may be fundamental in limiting the breakdown of joint cartilage.

MATERIALS AND METHODS

Bovine Nasal Cartilage Explant Cultures—Bovine nasal septa were obtained from a local abattoir and processed for culture within 2 h of slaughter. Full depth cores (from one perichondrium to the other) were immediately placed into cold Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin G and 100 μg/ml streptomycin. The tissue was washed twice with this mixture and once with fresh medium/BSA with 50 μM iodoacetamide in 25 mM NH₄HCO₃ and incubated in complete darkness at room temperature. The gel pieces were then washed three times with 40% (v/v) acetonitrile in 25 mM NH₄HCO₃ and evaporated to complete dryness.

Identification of Fibromodulin Fragment by Mass Spectrometry—Proteins were degraded into characteristic fragments with trypsin (Sequencing grade, Promega), endoproteinase Glu-C in phosphate-buffered saline (PBS), or endoproteinase Lys-C (Roche Applied Science). Briefly, gel pieces were re-hydrated in 12 μl of the respective enzyme solutions: trypsin at 20 μg/ml and Lys-C at 25 μg/ml in 25 mM NH₄HCO₃. Following re-hydration, 23 μl of the same buffer was added and samples were incubated at 37 °C for 16–24 h. For Glu-C digestion PBS was used instead of NH₄HCO₃. The gel pieces were then extracted with 2% (v/v) trifluoroacetic acid in water. Peptide containing solutions were then purified on C18 ZipTips (Millipore) with subsequent elutions at 20, 50, and 70% trifluoroacetic acid made up in saturated a-cyano-4-hydroxycinnamic acid (Sigma). One-microliter aliquots of elution solution/matrix mixtures were spotted directly onto a MALDI target plate and allowed to air dry.

The MALDI target plate was loaded into a Bruker Reflex™ III MALDI-TOF mass spectrometer. The polarity of the instrument was set for positive ions using delayed extraction and with the detector in reflector mode. The acceleration voltage was set at 26 kV and 120 shots per pixel were summed in each spectrum. Internal calibrants were used as reference masses. Spectra were analyzed for matches using the ProFound™ search engine (Rockefeller University) (35). Neo-epitope Antibody Production—The new N-terminal amino acid sequence Ala24-Pro25 was used to synthesize a peptide to which a C-terminal cysteine was added for coupling purposes. This peptide was coupled to BSA, keyhole limpet hemocyanin, and thiopropyl-Sepharose, respectively (Schäfer-N, Conways, Dentzer).

Five hundred micrograms of keyhole limpet hemocyanin conjugate was dissolved in 500 μl of PBS and then emulsified with an equal volume of complete Freund’s adjuvant. New Zealand White rabbits were injected with a 500-μg dose followed by a booster injection 4 weeks later. Booster injections were prepared in the same manner although with incomplete adjuvant. Test bleeds were taken 2–3 weeks after the final booster injection. Affinity Purification of Neo-fibromodulin Antiserum—A six-hundred-microliter (packed bed volume) column of neo-fibromodulin conjugated to thiopropyl-Sepharose was prepared in a Bio-Rad column fitted with a 22-gauge needle to slow down the flow rate. The column was washed with 4 ml of PBS. One milliliter of crude serum was allowed to drip through. The flow-through was recovered and passed through three times more. Four milliliters of PBS containing 1 μl NaCl was used to wash away loosely bound material. The column was then washed with 4 ml of PBS. Specific antibodies were eluted with 100 mM glycine-HCl, pH 2.8, and 250-μl fractions were neutralized by collection into Eppendorf tubes containing 12.5 μl of 100 mM Tris-HCl, pH 9.5.

Blots were blocked in 3% (w/v) low-fat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20 (Sigma) for 16 h at 4 °C. Fibromodulin was located using a rabbit polyclonal antisera specific for its core protein. The antibody was diluted in 1% (w/v) low-fat dry milk in Tris-buffered saline/Tween. The second antibody was a horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin (DAKO A/S, Denmark) also diluted in the same buffer. Reactive bands were visualized by incubating blots in the presence of a chemiluminogenic HRP substrate. Blots were exposed to CRONEXT™ film (Sterling Diagnostic Imaging).
Testing of Neo-epitope Antibody Specificity by Enzyme-linked Immunoassay—Falcon 96-well plates (BD Bioscience) were coated with 2 μg per well of either BSA-conjugated neo-fibromodulin peptide or purified bovine fibromodulin in 100 mM Na2CO3 buffer, pH 9.2, for 16–24 h at 4 °C. The plates were washed three times with 150 mM NaCl, 0.05% (v/v) Tween 20 (wash solution). Unoccupied sites were blocked with 100 μl of 1% (v/v) BSA in PBS for 30 min at ambient temperature. The plates were washed three times with wash solution. Affinity purified anti-serum was diluted to 1:100 in PBS, 1% (w/v) BSA, 0.05% (v/v) Tween 20 (PBS/BSA/Tween) and then doubling dilutions were carried out to 1:520,000. Test serum was incubated at room temperature for 1 h and then plates were washed three times with wash solution. The second antibody was a swine anti-rabbit alkaline phosphatase conjugated antibody (DAKO A/S, Denmark) used at 1:2,000 dilution in PBS/BSA/Tween and incubated for 1 h at ambient temperature. The plates were washed with wash solution and developed with 1 mg/ml p-nitrophenyl phosphate substrate in diethanolamine buffer, pH 9.8.

Testing of Neo-epitope Antibody Specificity by Western Blotting—Specificity for the cleaved form of fibromodulin and confirmation of the neo-epitope was performed by Western blotting of guanidine HCl extracts of cartilage cultured with IL-1. Each extract contains both intact and cleaved forms of fibromodulin, as judged by blotting with the anti-fibromodulin polyclonal antisera. Extracts were electrophoresed and transferred to nitrocellulose membranes. These were incubated with the anti-AYGSPPQPEP neo-epitope antibody at 1:250 in 1% skim milk for 3–4 h at room temperature. The conjugated second antibody was an anti-rabbit HRP (DAKO, Denmark) used at 1:1,000.

Generation and Purification of the N-terminal Peptide of Fibromodulin (Gln6–Lys16)—Purified bovine fibromodulin (36), 400–600 μg, was dissolved in 200 μl of Tris-buffered saline, pH 7.4, and 30 μl of 500 μg/ml Lys-C was added followed by incubation for 16 h at 37 °C. The reaction was stopped by addition 5 μl of 1 mg/ml TLCK and incubation for 30 min at 37 °C. N-Linked oligosaccharides were removed by addition of 5 units of N-glycosidase F (Roche) and incubation for 16 h at 37 °C. Before loading onto a MiniQ SMART column (Amersham Biosciences), 1500 μl of 10 mM Tris-Cl, pH 7.6, containing 6 M urea (buffer A for anion exchange chromatography) was added. A gradient of 0–100% buffer B (buffer A + 600 mM NaCl) over 20 column volumes was used to elute bound peptides. The column was regenerated with 2 ml of NaOH following each run. Aliquots from fractions containing peaks were dried and subjected to SDS-PAGE and Western blotting with the anti-fibromodulin polyclonal antisera and monoclonal antibody 5D4, directed against keratan sulfate chains. An aliquot from each fraction was desalted on C18 ziptips and peptides were eluted onto spotted dried α-cyano-4-hydroxycinnamic acid on a MALDI target plate.

Fractions deemed by Western blot and MALDI analysis to contain the N-terminal peptide were pooled and loaded onto a Sephasil C8 mini-RPC column (Amersham Biosciences). Bound material was eluted with a gradient of 0–100% acetonitrile in 20 column volumes. The resulting peak was dried and stored at −20 °C. Aliquots were taken from all steps of the digestion and purification processes and evaluated by Western blotting with an anti-fibromodulin polyclonal antisera and monoclonal antibody 5D4, directed against keratan sulfate chains (a kind gift from Dr. Bruce Caterson).

Determination of MMP Activity—One microgram of the 10-kDa N-terminal fragment of fibromodulin was added to 100 μl of conditioned culture medium from day 16. Possible combinations of presence or absence of the following additives were elaborated: CaCl2, 5 mM; aminophylline, 5 mM (stock solution 200 mM in methanol); monoclonal antibody 5D4, directed against keratan sulfate chains (Lys99), an aliquot of peptide found to be biotinylated by Western blotting was digested with trypsin and analyzed by MALDI-TOF MS.

Kinetics of Degradation in Conditioned Medium of the Biotinylated N Terminal of Fibromodulin—The biotinylated 10-kDa N-terminal fragment was dissolved in Tris-buffered saline, pH 7.4, 0.05% NaN3, and 5 μg was added to a 200-μl aliquot of conditioned culture medium from days 3, 6, 9, 12, 16, and 20, respectively. Fifty microliters from each respective sample was removed immediately and treated with EDTA at 15 mM final concentration. Removal of a 50-μl aliquot was repeated for each sample at 3, 6, and 20 h and the reaction was stopped with EDTA. A control consisted of 200 μl of Dulbecco’s modified Eagle’s medium, 100 μg/ml BSA, and 5 μg of biotinylated N-terminal fibromodulin. This sample was incubated for the full 20-h period. Laemmli loading buffer was added to the EDTA-treated samples and they were electrophoresed on 13% SDS-PAGE gels. Blotting was performed and blotted proteins were reacted with streptavidin-HRP at 1:5000 in 1% skim milk. The blots were developed with ECL reagent and exposed to membranes to CRONEX film.

Gelatin and Casein Zymography—Aliquots of conditioned medium from days 3, 6, 9, 12, 16, and 20 of culture were added with Laemmli loading buffer (1% SDS final concentration) and applied to casein and gelatin zymograms (Bio-Rad). Gels and buffers were cooled on ice and the current was fixed at 30 mA. SDS was scavenged from the gels with 2 changes of 2.5% (v/v) Triton X-100 in distilled water and one wash with water each for 15 min. The gels were incubated at 37 °C overnight in 15 ml of 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 10 mM CaCl2, 0.05% NaN3. The zymograms were then fixed in 40% ethanol, 7% acetic acid and stained with Coomassie Brilliant Blue R-250.

![FIG. 1. Fibromodulin is degraded and released before significant loss of type II collagen from cartilage explants. a, collagen content in explants with time in culture for control and IL-1-treated cultures. U, uncultured cartilage; 0, after 2 days pre-culture. Each point indicates the mean of 6 observations. Error bars indicate the standard deviation. b, lower panels show Western blots for fibromodulin in media at different times of culture in the presence or absence of IL-1a. Aliquots of culture media were precipitated with ethanol and treated with N-glycansase F. Samples were separated on 10% SDS-PAGE gels and transferred to nitrocellulose. Localization of fibromodulin was achieved using a polyclonal antibody specific for its core protein. The arrow at the left of each lower panel indicates the position of the cleavage product.](http://www.jbc.org/Downloadedfrom)
Cleavage of the N-terminal Peptide of Fibromodulin by Purified MMPs-2, -8, -9, and -13—To determine candidate metalloproteinases that perform the cleavage of interest, the N-terminal peptide of fibromodulin was incubated with purified MMPs-2 and -9 (Chemicon), recombinant rat MMP-8 (a kind gift from Dr. Chris Overall, University of British Columbia, Canada), and recombinant human MMP-13 (generously provided by Dr. Peter Mitchell, Pfizer, Groton). Incubations were performed at 37 °C in 50 mM Tris-HCl, pH 7.6, 5 mM CaCl₂, 10 mM Triton X-100, 0.05% NaN₃, at enzyme:substrate ratio of 1:20, 100, 500, and 2500, respectively. Aliquots were taken at 0, 3, 6, and 20 h and the protease was inhibited with 15 mM EDTA, final concentration. Western blots on these digests were blotted with either anti-fibromodulin polyclonal antiserum or streptavidin-HRP (DAKO, Denmark) when using the biotinylated N terminus of fibromodulin.

Characterization of the Cleavage Product Obtained by MMP-13—Ten micrograms of the N-terminal peptide were incubated with MMP-13 for 2 h at 37 °C in 50 mM Tris-HCl, pH 7.6, 5 mM CaCl₂, 0.05% NaN₃, at a 1:100 enzyme to substrate ratio. A control, without enzyme was incubated alongside. The reactions were stopped with EDTA. An aliquot was dried and subjected to limited trypsin digestion for 6 h. Identity of peptides in the digest was determined by MALDI-TOF mass spectrometry as outlined above. The digest was also separated on a Pharmacia C8 reversed phase column. An aliquot of each peak-containing fraction (no digestion with trypsin) was spotted directly onto a MALDI target plate and analyzed.

Western Blotting for MMPs—Blots for the presence of pro and active MMPs were performed using the following antibodies that reacted with their respective positive controls and showed species cross-reactivity with the bovine protein: MMP-2, mouse anti-human MMP-2, MAB3308 (Chemicon); MMP-3, rabbit anti-MMP-3, AB19150 (Chemicon); MMP-9, rabbit polyclonal IgG, SA-106 (BIOMOL Research Labs., Inc.); and MMP-13, sheep anti-mouse MMP-13, antibody L29/6 (a generous gift from Dr. Gillian Murphy) (37). The following second antibody conjugates were used where appropriate: rabbit anti-mouse HRP, swine anti-rabbit HRP, and rabbit anti-sheep-HRP, all from DAKO.

Blotting on nitrocellulose was performed as described above for Western blotting of fibromodulin. Blots on polyvinylidifluoride membranes (Pall, Inc., Portsmout, United Kingdom) were blocked with 4% fish gelatin for 2 h. Antisera used in blotting were diluted in 2% fish gelatin.

Treatment of Bovine Articular Cartilage Explants with Recombinant MMP-13—Bovine metacarpophalangeal joints were dissected to expose the articular cartilage. Full depth strips were removed from the subchondral bone with a scalpel. Two millimeter discs were punched from these strips with a homemade punch tool. Each disc weighed ~3 mg. Each disc was incubated in 75 μl of MMP buffer to which was added 0, 1, or 4 μg of recombinant MMP-13 (not aminophenylmercuric acetate activated). Samples were incubated at 37 °C for 24 h. Following incubation, the cartilage discs were washed twice with 500 μl of 150 mM NaCl, 50 mM NaOAc, pH 5.8. Duplicate discs were extracted with 4 μl GdnHCl, 50 mM NaOAc, pH 5.8, containing “Complete” protease inhibitor mixture (Roche) for 48 h at 4 °C and extracts were pooled. A portion of ethanol-precipitated extract was dried and resuspended in 50 μl of 50 mM Tris acetate, pH 8, containing 0.1% (w/v) SDS and boiled for 3 min. N-Glycosidase F digestion, electrophoresis, and Western blotting were performed at 37 °C with anti-neo-fibromodulin. The position of cleavage was determined by Western blot analysis of the cleaved form of fibromodulin with trypsin. The expected tryptic peptides of the intact and fragmented fibromodulin were shown in the inset. The sequence is corrected according to that obtained for bovine fibromodulin (51).

![FIG. 2](http://www.jbc.org/) Determination of the N-terminal sequence at the cleavage site of fibromodulin from nasal cartilage explants treated with IL-1α. a, SDS-PAGE 10% gel stained with Coomassie Brilliant Blue G-250 of N-glycanase F-treated culture medium from IL-1α-treated bovine nasal cartilage explants at day 18 of culture, concentrated 20-fold. Identity of protein bands labeled at the right of the figure was determined from MALDI-TOF analysis of trypsin and Glu-C digests of these bands. b, mass spectrum obtained by in-gel digestion of fragmented fibromodulin with trypsin. The expected tryptic peptides of both the intact and in vitro cleaved peptide masses are shown in the inset. The sequence and intact mass are those of the corrected sequence for bovine fibromodulin (51). x axis, m/z monoisotopic ion mass [M + H] (Da); y axis, signal intensity absolute intensity (a.i.). c, confirmation of the N-terminal sequence using an antibody against AYGSPPQPEP for the cleaved form of fibromodulin. Binding curve for the anti-neo-fibromodulin antibody, wells coated with 2 μg of AYGSPPQPEPC-BSA (●) and with 2 μg of purified bovine fibromodulin (○). Western blots (inset) are from a GdnHCl extract of IL-1α-treated cartilage containing both the intact and fragmented fibromodulin. Lane 1 blotted with anti-bovine fibromodulin polyclonal and lane 2 with anti-neo-fibromodulin. The arrow at the right shows the position of fragmented fibromodulin. d, schematic representation of the region N-terminal to the N-terminal cysteine loop of fibromodulin. Amino acids comprised in the sequence to which was raised the neo-epitope antibody are underlined. The position of cleavage is indicated with the vertical dashed line. The sequence is corrected according to that obtained for bovine fibromodulin (51).
performed as described above in "SDS-PAGE and Western Blotting of Tissue Extracts and Media."

RESULTS

In Vitro Cleavage of Fibromodulin in Bovine Nasal Cartilage Explant Cultures—The initial observation that fibromodulin was cleaved to a smaller form prior to complete dissolution of cartilage pieces around day 18 of culture prompted us to determine whether this event was initiated prior to a noticeable reduction in collagen by hydroxyproline content in the explants. Collagen content was observed to significantly diminish at the day 18 harvest point in IL-1-treated cultures, whereas that of the controls remained stable throughout the culture period (Fig. 1a). In contrast, the peak of fibromodulin fragmentation appeared around day 12 of culture and there was noticeable fragment formation at day 6 (Fig. 1b, lower panel). The integrity of the fibromodulin molecule was not affected in control cultures (upper panel). The cleaved product could not be isolated by GdnHCl extraction of fresh uncultured cartilage (data not shown). Aggrecan levels were highest in the media harvested at days 3 and 6, whereas very low at later times. This shows that release occurred between days 0 and 6 (data not shown).

Loss of fibromodulin from the explants was determined by Western blotting followed by densitometry, using GelPro™, to provide a semiquantitative estimate. The ratio of density on blots corresponding to fibromodulin over hydroxyproline decreased with time in IL-1-stimulated cultures. A significant loss from explants was observed already at day 6 at which time stimulated explants had ~75% of the amount observed in the controls (control, 409 ± 72 versus IL-1 stimulated, 298 ± 32 arbitrary units/μg of hydroxyproline, mean ± S.D.). This proportional loss increased by day 12 to about 50% (control, 319 ± 21 versus IL-1 stimulated, 161 ± 86 arbitrary units/μg hydroxyproline, mean ± S.D.). By day 18, only 10% of the amount of fibromodulin remained in the IL-1-treated explants compared with that in the control (control, 325 ± 24 versus IL-1 stimulated, 33 ± 31 arbitrary units/μg of hydroxyproline, mean ± S.D.).

Fibromodulin Fragment Identification and Characterization—To determine the identity of the faster migrating band reactive with the anti-fibromodulin polyclonal antibody, in-gel digestion followed by mass spectrometry was used. All major bands that stained with Coomassie Blue G-250 were identified (Fig. 2a). The sequence coverage of the band containing fragmented fibromodulin indicated that the absolute C terminus was present. Peptides up to Ile576 were identified with both Lys-C and Glu-C digestion and up to Arg869 were covered with trypsin cleavage.

Analysis by trypsin digestion and mass fingerprinting of the other strongly staining bands in proximity of the cleavage product demonstrated BSA at an apparent mass of 55 and 67 kDa for the two respective forms. Two prominent bands at 37–39 kDa were found to match the chitinase-like gp-39 previously identified as a glycoprotein secreted by human articular chondrocytes in monolayer or explant culture (38).

Sequence coverage was up to 40% in trypsin-digested samples. Low sequence coverage, about 10% obtained for Lys-C digests was probably because of the fact that it cleaves fibromodulin at relatively few sites therefore yielding large peptides that were not detected with the method used. In fact, only four peptides (considering zero missed cleavages) below the mass of 3500 Da can be obtained with Lys-C. We were able to detect all of these. In comparison, all of the 11 peptides detected in trypsin digests were equal to or below a mass of 3335 Da.

A not matched tryptic peptide that appeared at all times in samples of fragmented fibromodulin in MALDI-TOF spectra was in the range of 1198.5–1198.9 Da. It was calculated that a cleavage between Tyr85–Ala86 in addition to the trypsin cleavage at Arg4′–Asp5′ would produce this peptide (Fig. 2b). The ion of this peptide was isolated and post-source decomposition confirmed its identity.

A neo-epitope antibody raised to the synthetic peptide AY-GSPQQEPFC was found to be reactive only with the cleaved form of fibromodulin when used for blotting guanidine HCl extracts of IL-1-treated cartilage (Fig. 2c, inset). Total reactive fibromodulin as determined by blotting with an anti-fibromodulin polyclonal is shown in Fig. 2c, inset. Likewise, no reactivity of the neo-epitope antibody with intact fibromodulin was observed in solid phase binding curves (Fig. 2c), whereas it was reactive with a molar equivalent of peptide conjugate.

Generation of an N-terminal Peptide of Fibromodulin by Lys-C Digestion and Its Purification—The N terminus of fibromodulin up to Lys86 could be generated by Lys-C digestion. When chromatographed on Mono-Q, the Lys-C digest of fibromodulin eluted as multiple poorly resolved peaks (Fig. 3a) that when pooled eluted as a single well resolved peak on a C8 reversed phase column (inset). This partial resolution on reverse phase chromatography is likely attributable to a minimal influence of the multiple tryrosine residues that are heterogeneous sulfated.

Evaluation of the 10-kDa fibromodulin N-terminal peptide demonstrated that Lys-C (Fig. 3b) readily cleaved the Lys85, Tyr86 bond. Individual fractions appeared identical on SDS-PAGE and Western blotting as shown by their pooled appearance in Fig. 3b, lane 4. The peptide migrated with an apparent mass close to 20 kDa on SDS-PAGE. Slower migration of the peptide could not be explained by missed cleavages because no keratan sulfate could be detected in the preparation using antibody 5D4, Fig. 3b (lanes 1 and 2), lower panel.

The elution position of the N-terminal peptide of fibromodulin on both anion exchange and reversed phase chromatography, is not greatly different from that of the intact protein (around 400 mM NaCl and 50% acetonitrile, respectively). It is likely that the extended N-terminal tyrosine sulfate domain contributes to the larger apparent mass. MALDI-TOF analysis of the Coomassie Blue-stained band of the N-terminal peptide of fibromodulin yielded identical spectra as that of the mixture before electrophoresis. The small amount of a slower migrating contaminant remained uncharacterized.

Demonstration of Metalloproteinase Activity—Activity assays in which intact purified fibromodulin was added to conditioned culture media yielded disappointing results.Little cleavage was apparent and this in no way reflected what was observed in the cultures themselves. It was then recognized that the entire purified protein had been extracted with GdnHCl and that it was no longer associated with its principle partner in cartilage, type II collagen (1). It was therefore decided to use the N-terminal peptide of fibromodulin as a substrate for in vitro assays with conditioned medium and with pure MMP preparations.

Addition of the 10-kDa fibromodulin N terminus to conditioned culture medium from time points at which cleaved fibromodulin was present in cultured explants yielded cleavage of the substrate in the presence or absence of added calcium and with or without activation of MMPs with aminophenylmercuric acetate (Fig. 4). Conclusive evidence for metalloproteinase activity was obtained with addition of o-phenanthroline. In all instances, with addition of o-phenanthroline, the added substrate was not degraded. Also, it was evident that degradation of endogenous fibromodulin was less apparent with addition of the inhibitor.
Cleavage of Fibromodulin in Cartilage Explants

Biotinylation of the N-terminal Peptide of Fibromodulin for Use in Assays—Given the large number of active components observed on conventional zymography, a specific method was required to identify the MMP that cleaves the N-terminal peptide of fibromodulin. Theoretically, this peptide is an ideal substrate for oriented peptide. The protease cleaving the N terminus of fibromodulin is a metalloproteinase. Overnight incubations of the N-terminal peptide of fibromodulin in conditioned medium from nasal cartilage explants with IL-1 at day 16 of culture. Incubations were in the presence or absence of additives. SDS-PAGE 13% gels were transferred onto nitrocellulose and blotted with an anti-fibromodulin polyclonal antibody. The top panel shows the control, without addition of the N-terminal fibromodulin peptide (standard shown in lane marked Std, position indicated by arrowhead at right). The position of endogenous fibromodulin is shown by an arrow at the right of each blot. The lower panel shows incubations with addition of the N-terminal fibromodulin peptide.

was important to determine whether this reaction could be successfully performed at this single site. Selective N-biotinylation was confirmed by MALDI-TOF MS of a tryptic digest of biotinylated 10-kDa peptide. The absolute N terminus of this peptide, 1953 Da, gave a prominent signal, whereas no biotinylated form of this peptide was present (expected at 2289 Da). The product of interest was observed at 3110 Da. A proportion of non-biotinylated reactant was observed but this would in no way affect the assay.

Candidate Metalloproteinases—We tested some candidate metalloproteinases, among them two collagenases, MMPs-8 and -13, and two gelatinases MMPs-2 and -9. Only MMP-13 was capable of efficient cleavage (Fig. 5a, shown for MMP-8 and -13). In contrast MMP-8 was unable to perform the cleavage at the 1:500 enzyme:substrate ratio. However, it did cleave very poorly at a ratio of 1:20 and this with 20 h incubation as was observed for MMP-2 and -9 (not shown). Digestion with MMP-13 of the 10-kDa N-terminal peptide of fibromodulin at a 1:100 enzyme:substrate ratio yielded two major products on high performance liquid chromatography (Fig. 5b). These were characterized by MALDI-TOF MS to be the intact substrate peptide and the cleavage epitope-bearing product identical to that isolated from cartilage explants cultured with IL-1 (tryptic peptides: Ala<sup>64</sup>-Arg<sup>74</sup>, 1198 Da, neo-epitope; Asp<sup>75</sup>-Arg<sup>95</sup>, N-terminal Cys loop containing peptide, 2414 Da; Ala<sup>84</sup>-Lys<sup>98</sup>, entire cleavage product unprocessed by trypsin, 4030 Da (Fig. 5c)). The remainder of the tyrosine sulfate-rich region could not be found, therefore it is likely that it is further processed.

Kinetics of Cleavage of the Biotinylated Substrate—The biotinylated substrate was used to determine whether the kinet-
Aliquots were removed at 0, 3, 6, and 20 h and the protease was inhibited with 15 mM EDTA. The samples were boiled in SDS-reducing sample buffer and separated on a 15% SDS-PAGE gel. The proteins were transferred onto nitrocellulose and blotted with a polyclonal antibody to bovine fibromodulin known to react with its N terminus. The digest (Fig. 6a, the N terminus of fibromodulin was digested by MMP-13 for 2 h at 37 °C with an enzyme:substrate ratio at 1:100. The digest (—in) was made up to 600 mM NaCl, 6 M urea, in Tris-HCl, pH 7.6, and applied to a Pharmacia C8 reversed phase column. An identical control without enzyme was run in parallel and chromatographed in the same manner (*). All bands reactive with the neo-epitope-specific antibody recognizing an N-terminal sequence of A64YGSPPQPEP (Fig. 7). In explants incubated with 1 or 4 µg of MMP-13, a dose-dependent yield of this fragment was detected. No fragment could be detected in the remainder of the culture period. Band patterns were similar in both assays (Fig. 6b, open arrow marked with an asterisk). This band was also detectable on nitrocellulose.

Cleavage of Fibromodulin in Bovine Articular Cartilage Explants Treated with Recombinant MMP-13—To demonstrate in situ that fibromodulin could be cleaved at the appropriate site by the enzyme identified, fresh articular cartilage explants were treated with recombinant MMP-13. Overnight digestion resulted in the formation of the large fibromodulin fragment reacting with the neo-epitope-specific antibody recognizing an N-terminal sequence of A64YGSPPQPEP (Fig. 7). In explants incubated with 1 or 4 µg of MMP-13, a dose-dependent yield of this fragment was detected. No fragment could be detected in explants incubated in parallel without addition of the enzyme (Fig. 7, lane 1).

**DISCUSSION**

In joint disease, articular cartilage destruction appears to progress in a sequence of events progressively engaging different classes of molecules (39). To allow studies of this sequence of events and critical steps, we have utilized a model of explant culture of cartilage stimulated by IL-1. Such a model has been used to study and characterize events and critical steps, we have utilized a model of explant culture of cartilage stimulated by IL-1. Such a model has been used to study and characterize events and critical steps, we have utilized a model of explant culture of cartilage stimulated by IL-1. Such a model has been used to study and characterize...
widely used, particularly in studies of aggrecan (40, 41) and COMP (42) release. In further work it has been shown that loss of collagen from the tissue occurs considerably later (22). In the current work, corroborating results showing early aggrecan and later collagen release is provided. Furthermore, it is shown that the collagenous network is affected between major aggrecan and collagen release. Thus, fibromodulin, which in the tissue exists bound at the surface of collagen fibers (43), was cleaved and fragments were released at an intermediate stage. This may represent an early critical event affecting the collagen fibrillar network and may also lead to the exposure of sites where enzymes can cleave the collagen. The thermal instability of fibrillar collagen at body temperature will result in rapid unwinding after cleavage by collagenases, making the unfolded molecules susceptible to further breakdown by gelatinases (44). Thus hydroxyproline as a measure of collagen release is most likely an appropriate measure of any process that will affect the integrity of the molecule.

Analyses of the fragments produced demonstrated that cleavage occurred between the sequence PAY and AYG in the N-terminal part of bovine fibromodulin. In subsequent experiments using a 10-kDa fragment of the N-terminal part of fibromodulin, the same cleavage was observed both for using the medium from IL-1-incubated cartilage as the enzyme source and after digestion with purified MMP-13. In other experiments it was shown that cleavage at the same site occurred in a similar fragment of human fibromodulin incubated with MMP-13. This cleavage site is slightly different in sequence and cleavage occurs at PAY-TYG (data not shown). It is not clear to what extent the tyrosine residues that surround the cleavage site are substituted with sulfate. This is a relevant issue in view of the fact that the N-terminal part of fibromodulin contains a number of tyrosine sulfate residues (8).

An antiserum was raised to a peptide with the N terminus AYGSPPQPEP. These antibodies did specifically react with the appropriate fragment in the medium of IL-1-stimulated cartilage, whereas they did not react with intact fibromodulin. It is not likely that the antibodies will react with a peptide containing a tyrosine sulfate close to its N-terminal free end that should be part of the epitope. Whether cleavage depends on any of the tyrosine residues in the N-terminal portion of the fibromodulin being sulfated is not answered by the present study. It is, however, of interest to verify this in view of the data indicating that cleavage of aggrecan by aggrecanase (one or more of ADAMTS-1 (45), 4 (46), 5 (47), and 9 (48)) apparently is much more efficient if there is a substituent present close to the cleavage site (49, 50). In this respect it is likely that the presence of tyrosine sulfate residues will change the conformation of the polypeptide domain and thereby the character of the substrate site.

**Fig. 6.** Presence of active MMP-13 in culture medium correlates with kinetics of degradation of the 10-kDa substrate. *a,* time course (0, 3, 6, and 20 h) for digestion of the biotinylated N-terminal peptide of fibromodulin in conditioned medium harvested at 3–4-day intervals from bovine nasal cartilage explants cultured in the presence of IL-1. Western blotting was performed with streptavidin-HRP. *b,* standard gelatin zymography of culture medium from nasal cartilage cultures treated with IL-1. *c,* Western blotting for MMP-13 of medium from days 3 to 20 of culture. The right-hand lane marked *+ve* denotes the positive control, human recombinant MMP-13. *Closed arrows* at the right indicate bands reactive with the antibody that are found in the positive control. The *arrowhead* indicates the position of active MMP-13. The *open arrow* marked with an asterisk indicates the position of a band that is not accounted for in the positive control.

**Fig. 7.** Recombinant MMP-13 treatment of articular cartilage results in the appearance of the Ala64 neo-N terminus. Fresh bovine articular cartilage (~3 mg) was incubated for 16–24 h at 37°C with: no enzyme, lane 1; 1 µg of MMP-13, lane 2; or 4 µg of MMP-13, lane 3.4, Western blot using anti-neo epitope A64YGSPQPQPEP, and *B,* with a polyclonal antiserum to whole fibromodulin.
Cleavage of fibromodulin in the tissue where the molecule is bound to collagen fibrils and thereby possibly presents a particular structure. This is corroborated by the absence of cleavage of purified intact fibromodulin by either the medium from stimulated explants or MMP-13. However, by using a fragment of the N-terminal part of fibromodulin, apparently sufficient binding structure and its appropriate conformation allowed specific cleavage at the expected site. This indicates that the conformation of the protein in the tissue may guide enzymes to bind and specifically cleave the protein. These findings are supported by the observation that the fragment can be extracted from cartilage tissue treated with recombinant MMP-13. Likewise, it is apparent that the composition and possibly structure of the macromolecules of the fresh tissue treated with MMP-13 is different from that of IL-1-treated explants at the time of fibromodulin cleavage. In the latter case, all aggrecan has been released. It thus appears that the cleavage occurring upon cytokine treatment is a result of the appearance of MMP-13 by day 6 rather than a change of conformation of the substrate in the tissue.

Because activity depended on divalent cations, it was apparent that the enzyme was a metalloproteinase. In Western blotting, the presence of MMP-13 could be detected at the correct time. In further experiments it could indeed be shown that MMP-13 showed orders of magnitude higher activity toward the substrate than several other MMPs. The difficulty in isolating the remaining part of the tyrosine-sulfated region after in vitro digestions with MMP-13 indicated that this peptide was further processed or did not adhere to the reversed phase column. The 10-kDa substrate when subjected to in-gel digestion with MMP-13, yielded several other fragments. The high degree and apparent variability of tyrosine sulfation of this part of the N-terminal portion of fibromodulin (51) has precluded their identification.

Medium from points with high aggrecan release and therefore aggrecanase activity showed no ability to degrade the substrate. Future work will have to evaluate the ability of specific inhibitors for MMP-13 to block the formation of this fragment. Also such studies would be of value in attempting to prevent further destruction of cartilage, by e.g. breakdown of the collagen itself. An interesting issue is the comparatively late activation of MMP-13, well after the peak of aggrecan release that was high already at day 3 and had occurred before day 6 (data not shown). This demonstrates early and high aggrecanase activity. A question is what is activating the MMP and whether it is produced, but not activated prior to its peak activity.

The functional consequences of the cleavage observed remain to be elucidated. However, it is likely that binding to collagen depends on domains of the leucine-rich repeat region of the protein as has previously been observed for decorin (52, 53). The observed cleavage separates the N-terminal heavily negatively charged domain of fibromodulin from the portion binding to collagen. It appears that the main fragment is retained for a more extensive period in the tissue, apparently by its binding to collagen. The N-terminal domain of fibromodulin is likely to form a complex with surrounding molecules having domains with basic charge. Thus, removal of this portion of the protein is likely to result in weaker interactions of a particular collagen fiber to neighboring structures. This may result in altered mechanical stability. Furthermore, alterations of the collagen fibril surface in the way demonstrated may open up structures for further digestion by collagen-degrading enzymes.

Taken together the observed degradation is likely to be an important event in the progressive destruction of articular cartilage in joint disease. The identification of the cleavage site will allow development of procedures for its identification in various samples of joint disease tissues. The fact that the released N-terminal fragment contains a domain with variable tyrosine sulfation (51) has precluded development of an assay. This cleavage as a step in developing joint disease therefore remains to be corroborated. The fragment of fibromodulin retained in the tissue will be further degraded as tissue destruction proceeds.

The identification of a candidate enzyme will open up possibilities for specific inhibition in attempts to reverse or retard the induced cartilage degradation process. Our current data with no detectable presence of fragments containing the cleavage site now described in normal cartilage indicates that this cleavage is not significant in normal cartilage turnover, but rather a consequence of a pathological process. It is also notable that the observation that a collagenase will actually cleave a substrate. FEBS Lett. 11874–11880

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**Cleavage of Fibromodulin in Cartilage Explants**
Cleavage of Fibromodulin in Cartilage Explants Involves Removal of the N-terminal Tyrosine Sulfate-rich Region by Proteolysis at a Site That Is Sensitive to Matrix Metalloproteinase-13

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