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Regulation of Collagenase Activities of Human Cathepsins by Glycosaminoglycans*

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Cathepsin K, a lysosomal papain-like cysteine protease, forms collagenolytically highly active complexes with chondroitin sulfate and represents the most potent mammalian collagenase. Here we demonstrate that complex formation with glycosaminoglycans (GAGs) is unique for cathepsin K among human papain-like cysteine proteases and that different GAGs compete for the binding to cathepsin K. GAGs predominantly expressed in bone and cartilage, such as chondroitin and keratan sulfates, enhance the collagenolytic activity of cathepsin K, whereas dermatan, heparan sulfate, and heparin selectively inhibit this activity. Moreover, GAGs potently inhibit the collagenase activity of other cysteine proteases such as cathepsins L and S at 37 °C. Along this line MMP1-generated collagen fragments in the presence of GAGs are stable against further degradation at 28 °C by all cathepsins but cathepsin K, whereas thermal destabilization at 37 °C renders the fragments accessible to all cathepsins. These results suggest a novel mechanism for the regulation of matrix protein degradation by GAGs. It further implies that cathepsin K represents the only lysosomal collagenolytic activity under physiologically relevant conditions.

Controlled degradation of collagen is observed in bone remodeling, wound healing, angiogenesis, and during organ development (1–3). On the other hand excessive collagen degradation leads to pathological phenotypes such as osteoporosis (4), various forms of arthritis (5), or aneurysms of blood vessels (6), or it is characteristic for tumor invasion (7). However, triple helical collagens, in particular type I and II collagens, are highly resistant to general proteolysis and require specific proteases for their degradation. Known mammalian collagenolytic activities include members of the matrix metalloprotease family such as MMP-1, -2, -8, -13, and -14 (8), the serine protease, human neutrophil elastase (9), and thiol-dependent cathepsins (1). Collagenases of the MMP family cleave triple helical collagen at a specific single site and release ¾ and ¼ fragments. Similar to MMPs, human neutrophil elastase generates ¾ fragments from type I collagen but is unable to degrade type II collagen (9). Lysosomal cysteine proteases such as cathepsins L and B have also been discussed as collagenolytic activities, but these data were mostly based on inhibitor experiments in cell extracts or on early preparations of cathepsins, which may not have excluded contaminating activities (10–13). Thorough enzymatic studies suggested that cathepsins B and L primarily cleave in the non-helical telopeptide extensions of collagens (14, 15). A truly triple helical collagenase activity is found in cathepsin K, which is predominantly expressed in osteoclasts and to a lower degree in various other cell types including fibroblasts (16–19). It was demonstrated that cathepsin K, similar to the bacterial *Clostridium* collagenase, cleaves at multiple sites within the triple helical region of types I and II collagens (20, 21). The biological relevance of the collagenolytic activity of cathepsin K was underlined by the finding that deficiency in this protease causes the bone-sclerosing dysplasia, pycnodysostosis, in man (22) and an osteopetrotic phenotype in mice (23). The microscopic phenotype of pycnodysostosis is characterized by the accumulation of undigested collagen fibrils in osteoclasts (24) and other collagen-degrading cells such as fibroblasts (25). The accumulation of collagen fibrils in cathepsin K-deficient fibroblasts throughout the body suggests a broader role of cathepsin K in collagen turnover than previously thought and is, thus, not only limited to the osteoclast-mediated bone collagen degradation. The inhibition of cathepsin K in synovial fibroblasts, which are considered as the main cartilage-destroying cells in rheumatoid arthritis (26), also leads to the appearance of intralysosomal collagen fibrils (27). Recently, we have shown that the collagenase activity of cathepsin K depends on the formation of a complex with bone- and cartilage-resident glycosaminoglycans (GAGs)[1] such as chondroitin sulfate (28). To better understand the collagen catabolism and its regulation, we investigated the interactions of various GAGs with physiologically relevant human cathepsins and their abilities to degrade triple helical collagens. We demonstrate that only cathepsin K forms collagenolytically active complexes and that its collagenase activity and those of other cathepsins is regulated by individual glycosaminoglycans.

EXPERIMENTAL PROCEDURES

Enzymes—Human cathepsins K, L, and F were expressed in *Pichia pastoris* as described in (29, 30), and cathepsin S was expressed in Sf9 cells using the baculovirus expression system (31). Recombinant human cathepsin B was a generous gift from Dr. Robert Menard (Biotechnology Research Institute, Montreal, Quebec, Canada). Chondroitin-6 sulfate (C-4S), C-6S, dermatan sulfate (DS), keratan sulfate (KS), heparan

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[1] The abbreviations used are: GAG, glycosaminoglycan; C-4S, chondroitin-4 sulfate; C-6S, chondroitin-6 sulfate; DS, dermatan sulfate; KS, keratan sulfate; HS, heparan sulfate; DTT, dithiothreitol; E64, L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butanell; MMP, matrix metalloproteinase; DCG-04, biotin and tyrosine modified E-64 derivative; LHVS, morpholine urea-leucyl-homophenylalanyl-(vinylsulfonyl)benzene.

sulfate (HS), and heparin were purchased from Sigma. C-4S was fractionated on a Sephadex G-75 Superfine column, and the molecular masses of the individual fractions were determined by dynamic light scattering (28). Molar concentrations of active cathepsins K, B, L, and S were obtained by titration with E64 (32), and that of cathepsin F was obtained with the irreversible inhibitor, LHVS (kindly provided by Celera Corp, South San Francisco, CA) using the same method as described for E64. Human recombinant collagenase 1 (MMP-1) was cloned from human smooth muscle cDNA (Clontech, Palo Alto, CA) using the following primer pair: 5′-CGTCATATGTTCCCAGCGACTCTAG and 5′-CGCCAGATCTTCAATTTTTCCTGCAG by polymerase chain reaction using *Pfu* polymerase. A 1.4-kbp PCR product was obtained and subcloned into pGEM-T vector (Promega, Madison, WI), digested with NdeI/BglII and cloned into the NdeI/BamHI sites of the pET16b vector (Novagen, Madison, WI)). The sequence of the final plasmid was confirmed by automated DNA sequencing. BL21[DE3] cells transformed with MMP-1/pET16b were grown at 37 °C to an A_{600} of 1 and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. Cells were harvested by centrifugation and treated with lysozyme (1 mg/ml) in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and sonicated on ice. The washed pellet was resuspended in denaturing buffer (100 mM NaH_2PO_4, pH 8.0, 10 mM Tris, 8 M urea) and incubated at room temperature for 30 min by gently vortexing. After centrifugation the supernatant was applied on nickel nitrilotriacetic acid resin equilibrated with the same buffer and incubated at room temperature for 1 h. Unbound proteins were washed out with washing buffer (100 mM NaH_2PO_4, pH 5.9, 10 mM Tris, 8 M urea). MMP-1 was eluted with elution buffer (100 mM NaH_2PO_4, pH 4.5, 10 mM Tris, 8 M urea) and diluted into folding buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM $CaCl_2$, 50 μM $ZnSO_4$, 0.05% Brij35, 2.5 mM GSSG, 2.5 mM GSH, and 20% glycerol) to a final concentration of 4 M urea. The refolding reaction was carried out at 4 °C overnight. The urea concentration was reduced to 2, 1, 0.5, and 0 M by dialysis against dialysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35). Pro MMP-1 was activated by treating with 1 mM 4-aminophenylmercuric acetate at 37 °C for 30 min. 150 μg of purified enzyme was obtained from a 100-ml culture.

Size Exclusion Chromatography—40 μg of purified recombinant human cathepsins K and L were separately preincubated with 0.1% C-4S (29.8-kDa fraction) in the elution buffer for 20 min and then applied onto a Superdex 200 column and eluted either in the presence or absence of 300 mM NaCl with 100 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA and 1 mM dithiothreitol (DTT). To determine the competitive binding of C-4S and DS to cathepsin K, the protease was incubated with 0.1% DS or with a mixture of C-4S and DS, each at 0.1% concentration for 20 min, and then separated on Sephadex 200. Protein elution was monitored at 280 nm, and cathepsin activities were assayed for the hydrolysis of the fluorogenic substrate, benzyloxycarbonyl-Leu-Arg-7-amino-4-methylcoumarin as previously described (17).

Labeling of Cathepsins and Complex Formation with Chondroitin Sulfate—Cathepsins K, L, B, S, and F were labeled with ^{125}I-labeled DCG-04 as previously described (28). DCG-04 is an epoxide inhibitor derivative of E64 containing a tyrosine residue for iodination and a biotin moiety (33). 50 ng of ^{125}I-labeled DCG-04 cathepsins were preincubated in 100 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 1 mM DTT in the presence or absence of 0.1% of C-4S, respectively, for 20 min and then mixed at 37 °C with non-reducing protein loading buffer and preheated agarose gel. The sample was loaded into the well of a 0.5% agarose gel and analyzed as previously described (28). To determine potential complex formations of cathepsin K with other glycosaminoglycans, DCG-04-labeled cathepsin K was incubated in the presence of C-4S, C-6S, DS, KS, HS, or heparin and analyzed as described above.

Collagen and Gelatin Digests—0.4 mg/ml type I collagen (calf skin, Calbiochem) or 0.4 mg/ml type II collagen (calf articular joints, Amersham Biosciences) were incubated with human cathepsins K, B, L, S, or F (each cathepsin at 800 nM) in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT and EDTA. Collagen digestion was performed at 28 and 37 °C for 8 h in the absence and the presence of 0.15% (w/v) C-4S. Heat-denatured type I collagen (gelatin) was incubated with cathepsins (each at 2 nM) for 4 h at 28 °C. For the analysis of the effect of different GAGs on the collagenolytic activity of cathepsin K, recombinant human cathepsin K (800 nM) was incubated with 0.4 mg/ml type I collagen in the presence of 0.15% C-4S, C-6S, DS, KS, HS, or heparin at 28 °C for 8 h. To study the competitive effect of GAGs on cathepsin K-catalyzed collagen degradation, 0.15% C-4S in the digest mixture was successively replaced with increasing amounts of DS, HS, or heparin to reach a final concentration of 0.15%. All digest reactions were stopped by the addition of 10 μM E64 or LHVS in the case of cathepsin F. Samples were subjected to SDS-polyacrylamide electrophoresis using 4–20% Tris/glycine gels that subsequently were stained with Coomassie Blue.

Degradation of Insoluble type I Collagen and Cartilage—4 mg/ml insoluble bovine collagen of type I (Achilles tendon, Sigma) and 45 mg of finely minced bovine cartilage pieces (~1 mg) (Animal Organ and Tissues for Research, NJ) were washed 3 times with 100 mM acetate buffer, pH 5.5, and incubated with recombinant cathepsins K, L, B, S, or F (each 800 nM) in 900 μl of 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT/EDTA in the absence or the presence of 0.15% C-4S at 28 °C for 24 h. After the completion of the incubation time, 100 μl of supernatant were taken and hydrolyzed in 6 N HCl. The hydroxyproline content of soluble collagen fragments was measured according to the method described by Firschein and Shill (34) and as previously described (28). Analogous experiments were performed with cathepsin K in the presence of various glycosaminoglycans such as C-6S, DS, KS, HS, or heparin (0.15% each).

For SDS-polyacrylamide electrophoresis analysis, predigestions of 25 mg of insoluble type I collagen with recombinant human MMP-1 (400 nM) were performed at room temperature for 2 days in 0.5 ml of 100 mM acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. Subsequently, the reaction mixture was centrifuged, the supernatant was substituted with 2.5 mM DTT and 20 mM EDTA, and the MMP-1-digested samples were incubated with cathepsins K, L, B, S, or F (each 800 nM) at 28 or 37 °C for 8 h in the presence of 0.15% C-4S. The reactions were stopped by the addition of 10 μM E-64 after 24 h, and aliquots of the supernatant were taken and subjected to SDS-polyacrylamide electrophoresis using 4–20% Tris/glycine gels that subsequently were stained with Coomassie Blue.

Circular Dichroism Spectroscopy—Type I collagen was dissolved in 5 mM aqueous acetic acid at a final concentration of 50 μg/ml and clarified by centrifugation. In one set of experiments the soluble collagen sample was either analyzed directly or treated with 0.1% C-4S and analyzed. In a second set of experiments, 100 μl of the soluble collagen sample was either treated with 2 μl of enzyme buffer (50 mM Tricine, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij35) and analyzed or treated with 2 μl of 1 nM MMP-1 (dissolved in enzyme buffer) overnight at 37 °C, stored at 4 °C for 48 h, and analyzed. CD spectra were recorded over the range $\lambda = 190–250$ nm on a Jasco J-600 using a 10-mm path length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity ($[\Theta]$) at $\lambda = 225$ nm while the temperature was continuously increased in the range of 5–80 °C at a rate of 0.2 °C/min. Temperature was controlled using a Jasco PTC-348WI temperature control unit. For samples exhibiting sigmoidal melting curves the reflection point in the transition region (first derivative) is defined as the melting temperature (T_m).

Electron Microscopic Analysis of Cartilage Degradation by Cathepsins and MMP-1—Bovine cartilage disc were incubated with cathepsin K, with a mixture of cathepsins L and B, with MMP-1, or with a mixture of MMP-1 and cathepsin L and B, respectively, for 24 h at 37 °C. The cathepsin concentrations were 800 nM, and the concentration of MMP-1 was 400 nM in the assays. The digestion experiments with cathepsins were performed in 100 mM acetate buffer, pH 5.5, containing 2.5 mM DTT/EDTA, whereas digestions with MMP-1 alone or consecutively with cathepsins B and L were performed in 100 mM Tris·HCl buffer, pH 7.5, containing and 10 mM $CaCl_2$. Before incubation with cathepsins B and L, the Tris buffer was exchanged with 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT/EDTA. Following the enzymatic digest, cartilage specimens were immersed in a solution containing 3% glutaraldehyde with 0.2 M sodium cacodylate at pH 7.4. After overnight fixation the fixative solution was removed and replaced with phosphate buffer followed by 1% osmium tetroxide buffered with sodium cacodylate. After 1 h the osmium was replaced with increasing concentrations of ethanol. Cartilage samples were then placed in the critical point drier where the alcohol was exchanged for liquid CO_2. The CO_2 was removed at the critical temperature and pressure and coated with a light coat of gold palladium. The specimen was then mounted on an aluminum stub and viewed with a S-530 Hitachi S.E.

RESULTS

Lack of Complex Formation for Cathepsins Other Than Cathepsin K and Complex Formation of Cathepsin K with Other Physiologically Relevant Glycosaminoglycans—We have previously reported that human cathepsin K forms a high molecular mass complex in the presence of C-4S. This complex was demonstrated by gel filtration and in a gel mobility shift assay (28).

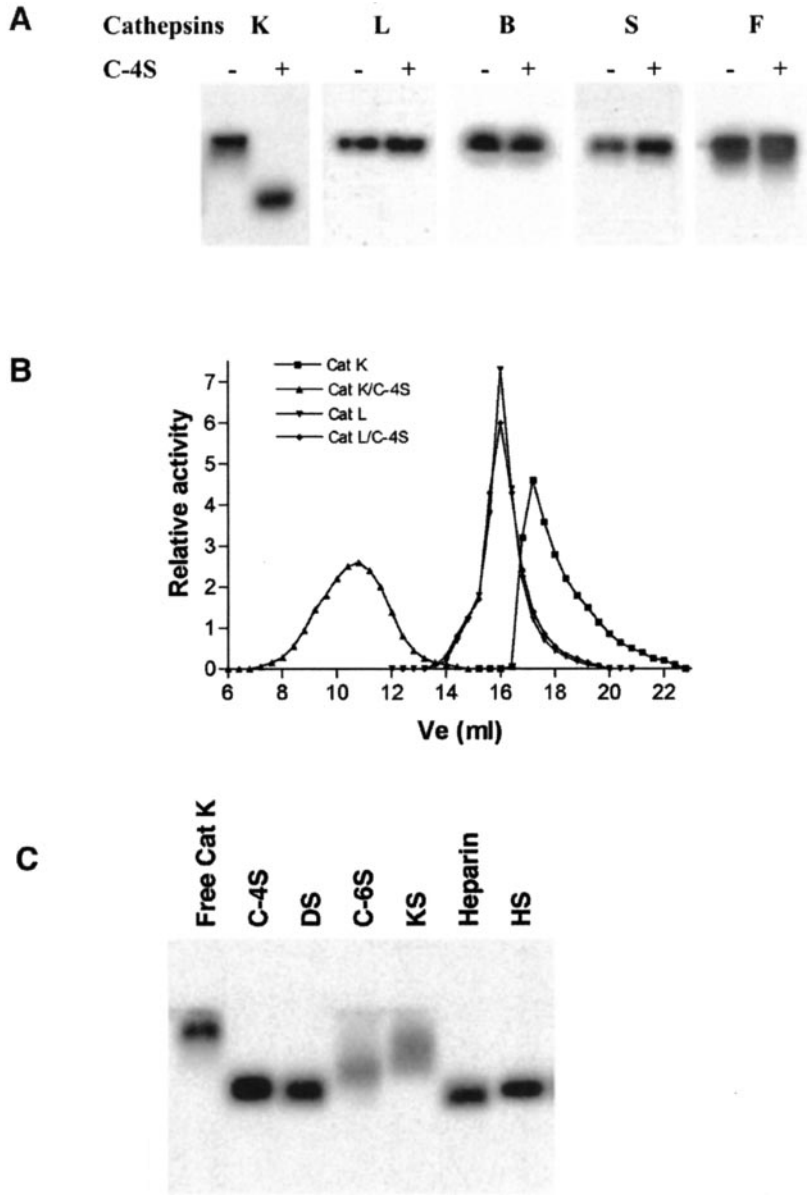


FIG. 1. *A*, electromobility shift assay of ^{125}I-labeled DCG-04-labeled cathepsins K, L, B, S, and F in the presence and absence of C-4S. Only cathepsin K forms a complex with C-4S. *B*, gel filtration assay with cathepsins (*cat*) K and L in the absence and the presence of C-4S using Superdex 200. Whereas cathepsin K elutes as a high molecular mass complex in the presence of C-4S, there is no difference in the elution profile for cathepsin L in the presence or absence of C-4S. *C*, electromobility shift assay of [^{125}I]-DCG-04-labeled cathepsins K in the presence of C-4S, DS, C-6S, KS, heparin, and HS. All glycosaminoglycans form complexes with cathepsin K.

Here we demonstrate that complex formation is unique for cathepsin K among closely related lysosomal cysteine proteases. Fig. 1 shows the absence of complex formation for cathepsins B, L, S, and F in the presence of C-4S (using the gel mobility shift assay, *panel A*) and the lack of a high molecular weight component in the gel filtration assay as demonstrated for cathepsin L (*panel B*). Cathepsin L activity eluted from the Superdex 200 column with an apparent molecular mass of 35 kDa irrespective of whether C-4S was present or absent in the preincubation mixture. In contrast, cathepsin K eluted with a molecular mass of 310 kDa when preincubated with C-4S (29.8 kDa) and as a 25-kDa protein in the absence of C-4S (*panel B*). Although the complex formation between cathepsin K and C-4S seems to be unique among cysteine proteases, C-4S is not the only GAG capable of binding to cathepsin K. In addition to C-4S, cathepsin K binds to a variety of GAGs such as C-6S, DS, KS, HS, and heparin (Fig. 1*C*). In contrast, none of these polysaccharides binds to cathepsin L (data not shown).

Degradation of Type I and II Collagens by Lysosomal Cathepsins in the Presence or Absence of C-4S—Type I and II collagens were incubated at 28 °C and at a body temperature of 37 °C in the presence or absence of C-4S with cathepsins K, B, L, S, and F (Fig. 2). As previously shown (35), the collagenolytic activity of cathepsin K is dramatically enhanced in the presence of C-4S. The complete degradation of both types of collagen at 37 °C by cathepsin K in the apparent absence of C-4S is due to contamination with GAGs in commercially available collagen preparations as previously demonstrated. The addition of 0.3 M NaCl, which prevents the formation of cathepsin K/C-4S complexes, also prevents the collagenolytic activity of cathepsin K at body temperature (28). Contrary to cathepsin K, recombinant human cathepsins B and F did not reveal any detectable collagenase activities at both temperatures in the presence or the absence of C-4S. The relative amount of the β and γ bands did not significantly change in the presence of both enzymes, suggesting only a weak, if any telopeptide-cleaving activity. However, cathepsins S and L displayed weak to moderate triple-helical collagenolytic activities at 28 °C and a strong collagenolytic activity at 37 °C in the absence of C-4S, suggesting a different cleavage mechanism. Surprisingly, the addition of C-4S to the digest mixture strongly suppressed the collagenolytic activity of both enzymes even at 37 °C. This reveals that individual GAGs can have opposite effects on the collagen-cleaving capability of closely related cysteine proteases, *i.e.* the activation of cathepsin K activity and the inac-

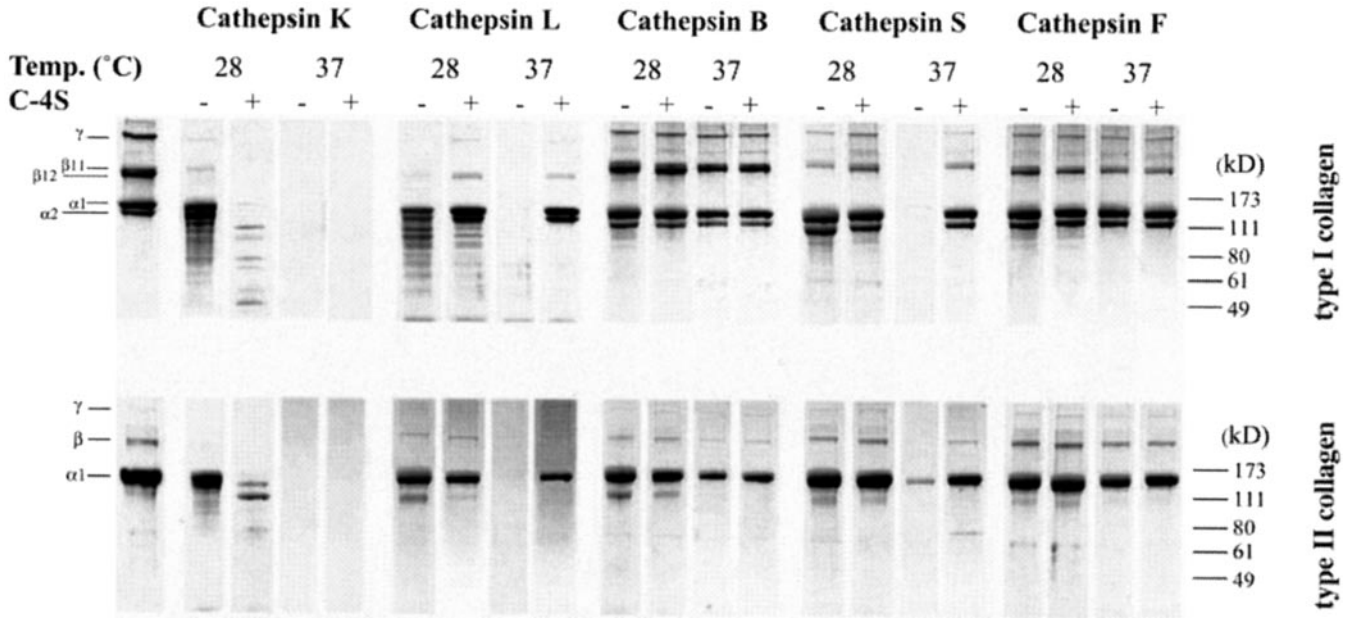


FIG. 2. **Triple helical type I and II collagen degradation by cathepsins K, L, B, S, and F in the presence or the absence of C-4S at 28 and 37 °C.** Both collagens are efficiently degraded by cathepsin K, whereas their degradation by cathepsins L and S is inhibited by C-4S at 37 °C. Cathepsins B and F do not exhibit any detectable collagenase activity.

tivation of cathepsins S and L activities, and thus, suggesting a specific mode of protease regulation.

The collagenase activity of cathepsin K in the presence of C-4S and the inhibition of the collagenase activities of cathepsins L and S was also reflected in degradation assays using insoluble type I collagen and cartilage (containing mostly type II collagen) (Fig. 3). The release of hydroxyproline-containing peptides from insoluble type I collagen into the supernatant of the reaction mixture increased more than 10-fold for cathepsin K in the presence of C-4S, whereas the activities of cathepsins L and S decreased 10 and 5 times, respectively. Similar to the activities toward soluble collagens, the activities of cathepsin B and F were negligible for insoluble collagen and not affected by the absence or presence of C-4S. Type II collagen in cartilage was exclusively degraded by cathepsin K with only a small increase of activity in the presence of C-4S. This can be attributed to the high concentration of GAGs, in particular chondroitin sulfates in the cartilage matrix. It should be noted that cathepsins could release up to 2.5 mg/ml GAGs from cartilage (data not shown). The major source of chondroitin sulfate is aggrecan, an abundant proteoglycan in cartilage. The inhibitory effect of cartilage-resident chondroitin sulfate on other cathepsins is reflected by the low activities of cathepsins L and S irrespective of the addition of exogenous C-4S. The hydroxyproline-releasing activities of both enzymes are similarly as low as those of the non-collagenolytic cathepsins B and F.

Surprisingly, recombinant human cathepsin B did not show any collagenase activity in our experiments either in the absence or the presence of GAGs nor at 37 °C, which is in contradiction to older reports (11, 14). Because previously enzyme preparations from tissues were used, it can be speculated that they contained small but sufficient contaminations of cathepsins L or K, accounting for the observed collagenase activities. On the other hand, the degradation of denatured type I collagen (gelatin) by any of the cathepsins tested was not affected by the presence of C-4S, suggesting that C-4S does not block or otherwise influence the catalytic site of the proteases (Fig. 4).

Consecutive Degradation of Type I and II Collagen with MMP-1 and Cathepsins—Collagenases of the matrix metalloproteinase family have been implicated in the extracellular degradation of triple helical collagens at neutral pH. Here, we

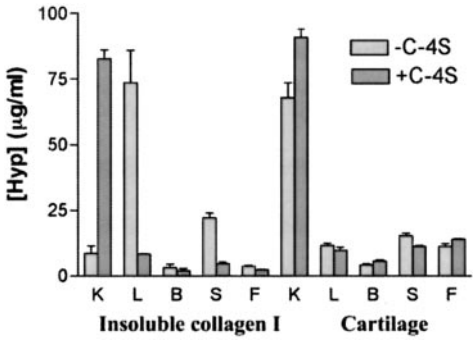


FIG. 3. **Comparison of the relative hydrolysis rates of insoluble type I collagen and bovine cartilage by cathepsins K, L, B, S, and F in the presence or absence of C-4S.** The collagenase activity was measured by determining the content of hydroxyproline in soluble collagen fragments. Exogenously added C-4S and cartilage resident glycosaminoglycans increase the collagenolytic activity of cathepsin K and inhibit the appropriate activities of cathepsins L and S.

investigated whether MMP-1-mediated predigestion of type I and II collagens permits the further degradation by lysosomal cathepsins at acidic pH in the presence of C-4S. MMP-1 cleaves both types of collagen at a single peptide bond within the appropriate α-chains and releases so-called ¾ and ¼ fragments. These fragments are only degraded by cathepsin K at 28 °C, whereas all other cathepsins tested were unable to further cleave these fragments in the presence of C-4S at this temperature (Fig. 5; data shown only for type I collagen). However, at 37 °C all cathepsins were capable of hydrolyzing the fragments in the presence of C-4S, indicating that the triple helical structure of the collagen fragments is sufficiently destabilized to allow complete degradation (Fig. 5). Similar results were obtained using cartilage or soluble type I and II collagens (data not shown).

Thermal Stability of Type I Collagen in the Presence or Absence of C-4S and after Digestion with MMP-1—The thermal stability of type I triple helical collagen is not influenced by C-4S, as demonstrated by the identical melting curves of collagen in the absence and presence of C-4S (Fig. 6*A*). This suggests that binding interactions between collagen and GAGs as described by

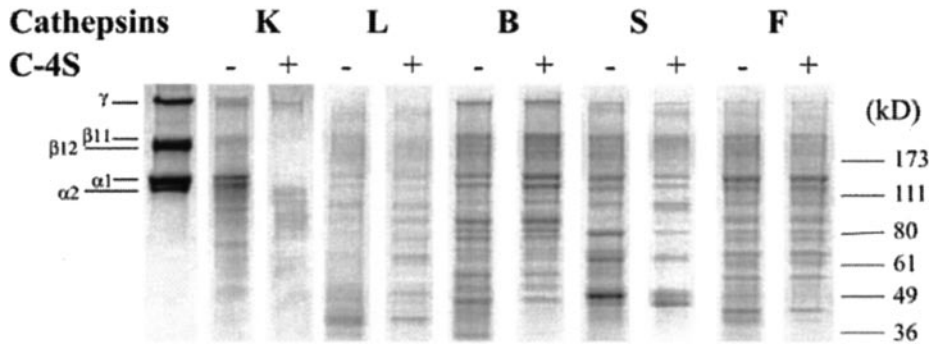


FIG. 4. **Comparison of the gelatinolytic activities of cathepsins K, L, B, S, and F in the presence or the absence of C-4S.** There is no significant effect of C-4S on the gelatinolytic activities of all cathepsins tested.

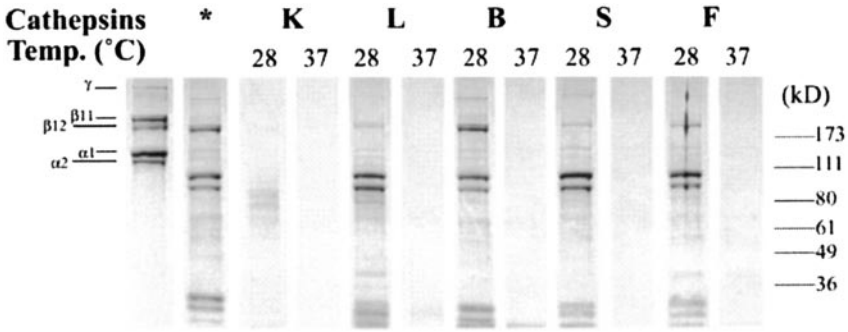


FIG. 5. **Degradation of MMP-1 generated type I collagen fragments by cathepsins K, L, B, S, and F at 28 and 37 °C in the presence of C-4S.** MMP-1-generated collagen cleavage fragments are stable against cathepsin activities tested except cathepsin K at 28 °C but are effectively degraded by all cathepsins at 37 °C. *, digest with MMP-1 alone.

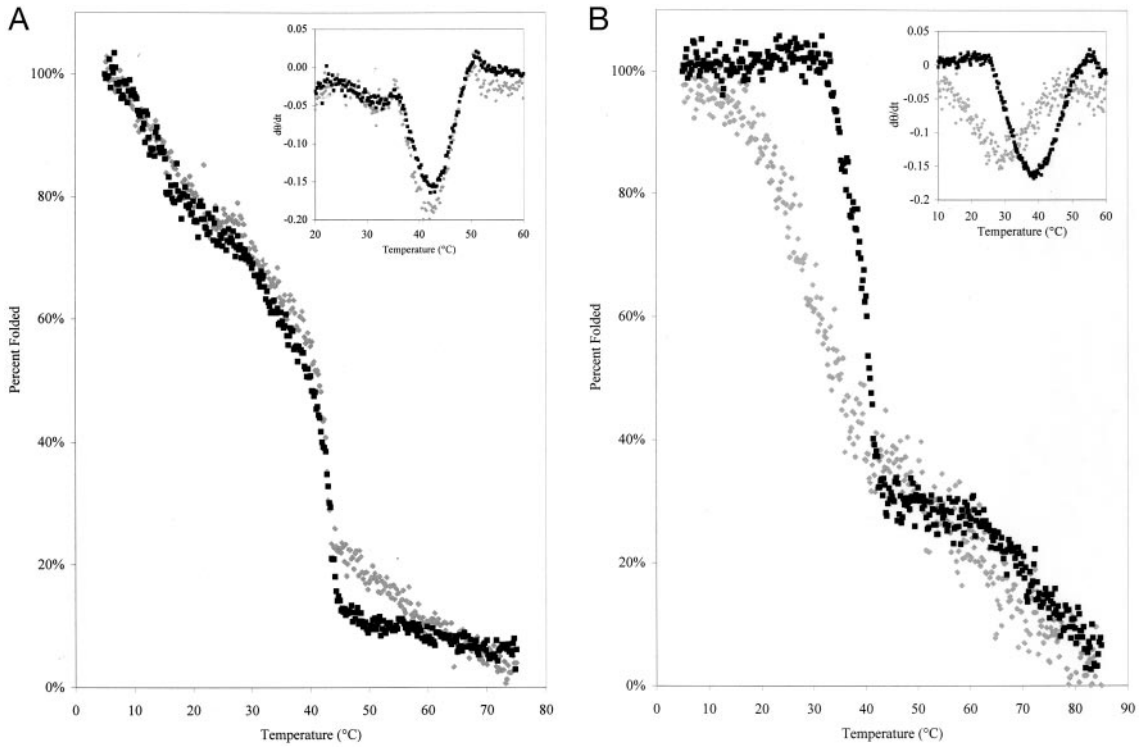


FIG. 6. **Circular dichroism spectra of type I collagen untreated (*black symbols*) or treated with 0.1% C-4S (*gray symbols*) (*A*) and type I collagen untreated (*black symbols*) or treated with 1 nM MMP-1 overnight at 37 °C (*gray symbols*) (*B*).** C-4S had no affect on the thermal stability of type I collagen, whereas MMP-1 treatment of type I collagen resulted in fragments of 8–10 °C lower thermal stability than the parent protein.

Komsa-Penkova *et al.* (36) do not significantly stabilize or destabilize the triple helical structure of the protein. However, the ¼ and ¾ polypeptide fragments of collagen generated by MMP-1 have significantly lower thermal stabilities than the parent protein. Here, the thermal stability of the fragments is 8–10 °C lower than type I collagen (Fig. 6*B*), which may explain the susceptibility of the degradation fragments to further cleavage by non-collagenolytic proteases (Fig. 5).

Electron Microscopic Analysis of Cartilage Degradation by MMP-1 and Various Cathepsins—Bovine cartilage discs were incubated with recombinant human cathepsins B, L, and K and with MMP-1 at different combinations at 37 °C. The experiments were performed 1) at pH 5.5 to reflect pH conditions observed in diseased cartilage (37) and within lysosomes or 2) at pH 7.5 to mimic the extracellular pH in normal cartilage and to provide optimal assay conditions for MMP-1. Because carti-

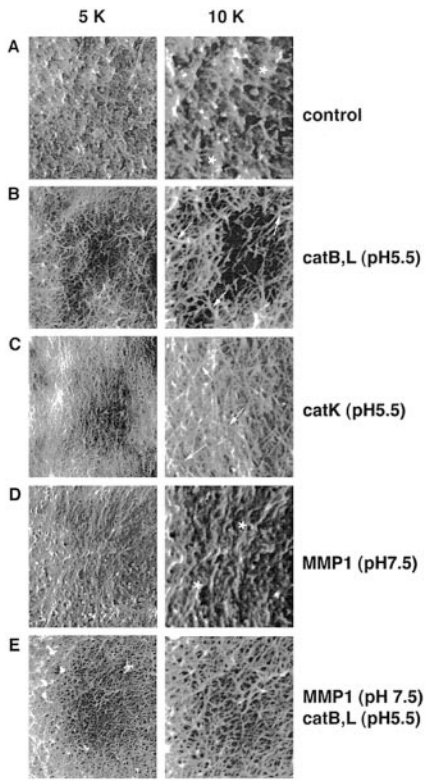


FIG. 7. **Scanning electron microscopic images of bovine cartilage treated with recombinant human MMP-1 and human cathepsins K, L, and B.** *Panel A*, control, untreated cartilage; *panel B*, incubation with cathepsins L and B at pH 5.5; *panel C*, incubation with cathepsin K at pH 5.5; *panel D*, incubation with MMP-1 at pH 7.5; *panel E*, incubation with MMP-1 at pH 7.5 and subsequent incubation with cathepsins L and B at pH 5.5. *Panels B*, *C*, and *E* reveal exposed collagen fibrils caused by cathepsin activities, whereas in *panels A* and *D* the fibril structure remains obscured by the interfibrillar matrix (see *asterisks*). The fibril thickness appears to be thinner in *panel C* (cathepsin K digested) when compared with *panel B* (cathepsins L and B digested; see *arrows* at 10,000× (*10 K*) magnification). The electron microscopy images were taken at a 5,000× (*5 K*) and 10,000× magnification. All cathepsin concentrations were 800 nM and that of MMP-1 was 400 nM. The digest reactions were performed at 37 °C for 24 h. Pictures taken from two independent digest experiments gave comparable electron microscopy images.

lage is rich in chondroitin sulfate (major glycosaminoglycan of aggrecan) the collagenolytic activity of cathepsin L is likely suppressed, as shown in Fig. 2. Thus, cartilage incubated with cathepsins B and L at pH 5.5 mostly reveals the removal of proteoglycan aggregates and the exposure of the collagen fibrils (Fig. 7, *panel B*). Incubation of cartilage with MMP-1 alone does not seem to alter the structure of the cartilage matrix when compared with the control (Fig. 7, *panels A* and *D*). This is in agreement with the absent or weak proteoglycan-hydrolyzing activity of MMP-1 and, thus, the inaccessibility of the collagen fibrils to MMP-1-mediated hydrolysis. In contrast, the incubation of the cartilage specimens with cathepsin K resulted in a complete exposure of the collagen fibrils and an apparent collapse of the fibril network and/or thinning of individual fibrils (Fig. 7, *panel C*). The simultaneous incubation of the matrix with MMP-1 and cathepsins B and L at pH 5.5 (not shown; acidic pH values have been described in diseased cartilage (37)) or the consecutive treatment of cartilage with MMP-1 at neutral pH followed by an incubation with cathepsins B and L at pH 5.5 (Fig. 7, *panel E*) do not differ significantly, suggesting that MMP-1 activity is also sufficiently active at acidic microenvironment conditions. Although the evaluation of the micrographs only allows a qualitative assessment of cartilage matrix alterations, it appears that the MMP-1/cathepsin double digests probably constitute an intermediate state of the matrix when compared with the cathepsins B/L and cathepsin K digests. Most likely, MMP-1-cleaved triple-helical collagen molecules still remaining within the fibril structure can only partially be degraded by cathepsins L and B. On the other hand, cathepsin K performing multiple cleavage sites within the triple-helical collagen units constituting the fibrils is more efficient than combinations of MMP-1 and other cysteine proteases other than cathepsin K.

Degradation of Type I Collagen and Gelatin by Cathepsin K in the Presence of Glycosaminoglycans Other Than C-4S—C-4S, the major glycosaminoglycan in cartilage and bone, is required for the efficient degradation of triple helical collagens by cathepsin K. However, various other GAGs are potentially in contact with cathepsin K as well, and their expression levels undergo significant changes during aging and disease. Thus, we evaluated the effect of those GAGs on the collagenolytic activity of cathepsin K. C-6S and KS, which are also present at high concentrations in cartilage, have a similar stimulating effect as C-4S (Fig. 8*A*). In contrast, DS, HS, and heparin strongly inhibited the collagenolytic activity of cathepsin K (Fig. 8*A*), whereas they do not significantly change the catalytic efficiency toward the synthetic cathepsin K substrate benzyloxycarbonyl-Leu-Arg-7-amino-4-methylcoumarin as previously reported (35). The absence of the collagenase activity cannot be attributed to a loss of stability of cathepsin K in the presence of DS, HS, and heparin since the residual activity of cathepsin K in the presence of those GAGs was comparable with those in the presence of C-4S and KS (data not shown). A similar inhibitory effect of DS, HS, and heparin toward the release of soluble collagen fragments was also observed using insoluble collagen fibrils (Fig. 9). The hydroxyproline content in the supernatant of the collagen fibril digests in the presence of DS, HS, or heparin was only slightly elevated to that in the absence of GAGs and ~6–7× lower than observed for the digests performed in the presence of C-4S and KS. The residual activity of collagenase activity measured in the apparent absence of GAGs might be due to minor but sufficient contaminations of commercial collagen preparations with chondroitin sulfates as previously demonstrated (28). As expected, the various GAGs have no effect on the gelatinase activity of cathepsin K (Fig. 8*B*), indicating that all cathepsin K-glycosaminoglycan interactions specifically act on the collagenase activity of this protease.

Competition of GAGs for the Binding to Cathepsin K—Next we investigated whether GAGs can compete with each other for the binding to cathepsin K and whether this affects the collagenase activity of the protease. As shown in Fig. 10*A*, C-4S, DS, and HS cathepsin K complexes can be distinguished by their electrophoretic migration pattern in the mobility shift assay. DS and HS complexes migrate further to the anode than the C-4S cathepsin complex. Interestingly, GAGs inhibiting the collagenase activity of cathepsin K bind significantly stronger to the protease than C-4S. Less than 5% of DS, HS, or heparin (Fig. 10*A*; heparin data not shown) compared with the C-4S concentration are sufficient to displace C-4S in the protease complex. Only at a 99-fold excess of C-4S over "inhibiting" GAGs is the C-4S-cathepsin K complex formed. When C-4S and DS were mixed in a 1:1 ratio, only the DS-cathepsin K complex was observed when separated by gel filtration (Fig. 10*B*). Using different ratios between C-4S and the inhibiting GAGs in the type I collagen degradation assay as in the mobility shift experiment, significant collagen degradation was observed only at C-4S concentrations higher than 90% and DS, HS, or heparin concentrations lower than 10% (Fig. 10*C*; results for heparin not shown). These findings clearly suggest that the colla-

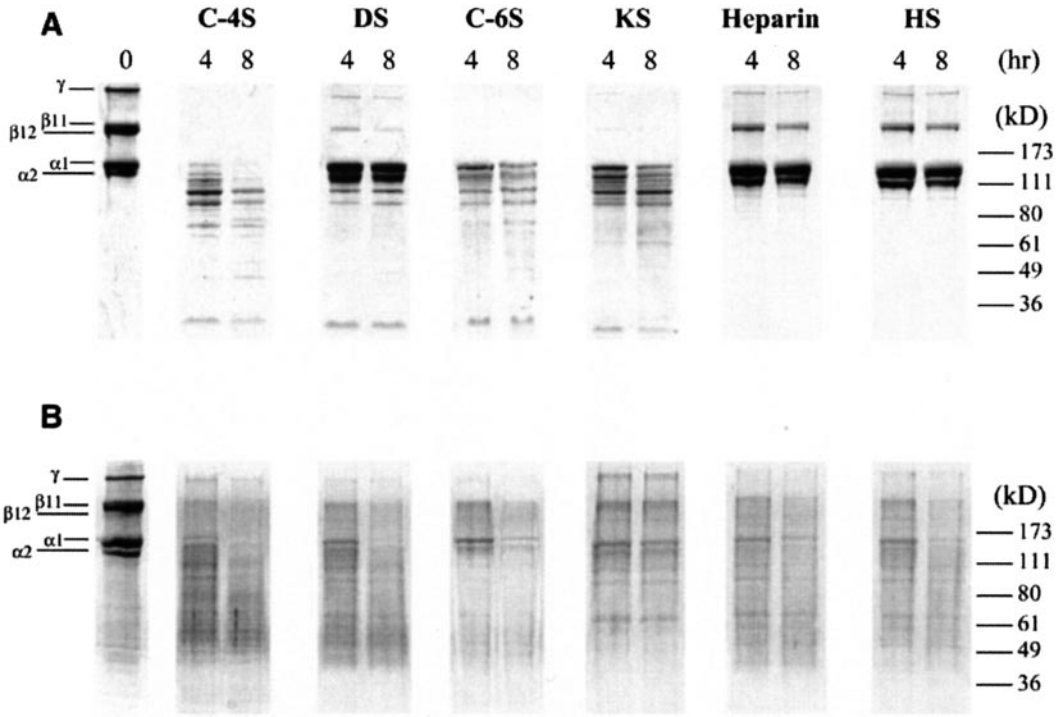


FIG. 8. **Degradation of type I collagen (*A*) and gelatin (*B*) by cathepsin K in the presence of C-4S, DS, C-6S, KS, heparin, and HS.** Major cartilage and bone resident GAGs such as C-4S, C-6S, and KS potentiate the collagenase activity of cathepsin K, whereas DS, HS, and heparin act as inhibitors.

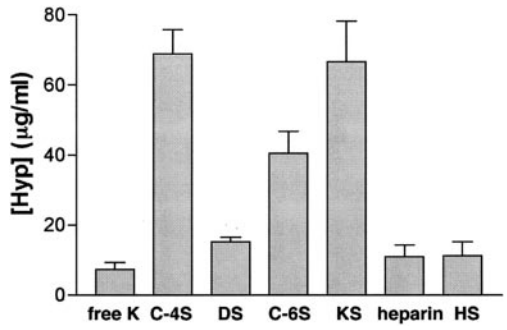


FIG. 9. **Comparison of the relative hydrolysis rates of insoluble type I collagen by cathepsins K in the presence C-4S, DS, C-6S, KS, heparin, and HS.** The collagenase activity was measured by determining the content of hydroxyproline (*Hyp*) in soluble collagen fragments.

genase activity of cathepsin K can be specifically modulated by individual GAGs.

DISCUSSION

The homeostasis of the extracellular matrix depends on the balance between the *de novo* synthesis and the degradation of matrix components. Major matrix components in connective tissue are collagens that require specific proteases for their degradation. We and others demonstrate that cathepsin K, a papain-like cysteine protease, is a highly potent collagenase capable to cleave triple helical collagens at multiple sites (20, 21). Although collagenolytic activities have been described for other cysteine proteases such as cathepsins B, L, and S (11, 38), cathepsin K appears to be by far the most active collagenase among them. This activity requires the formation of a unique complex between cathepsin K and chondroitin sulfate molecules. In the absence of chondroitin sulfate, cathepsin K is a monomer that lacks triple helical collagenase activity. None of the other cathepsins tested revealed the formation of C-4S-protease complexes. In contrast, cathepsin K can form appropriate complexes with any of the physiologically relevant GAGs. However, only a subset of GAGs allowed the generation of collagenolytically active complexes. Interestingly, these were GAGs that are predominantly expressed in bone and cartilage (chondroitin sulfates and keratan sulfates), the sites of the probably main biological activity of cathepsin K. These GAGs are easily liberated by cathepsin activities from aggregan aggregates or cartilage and can subsequently form complexes with cathepsin K (39). Other GAGs such as dermatan sulfate, heparan sulfate, and heparin are also able to form stable complexes but do not support any collagenase activity. However, these different complexes seem not to affect the catalytic site of cathepsin K since the Michaelis-Menten parameters for the hydrolysis of a synthetic substrate are not significantly altered in the presence of productive or non-productive GAGs (35). Moreover, the gelatinase activity of the various complexes is comparable with the monomeric cathepsin K form, suggesting that complex formation is specifically required for the degradation of triple helical collagens. Almeida *et al.* (40) recently demonstrated that interactions between cathepsin B and heparin or heparan sulfate potentiate the endopeptidase activity of the protease and that they also may stabilize the enzyme by reducing its loss of α-helix content. However, besides binding to a heparin column, no distinct complex between cathepsin B and GAGs was described.

Cathepsins B, L, and S have been described to cleave primarily in the non-helical telopeptide region of type I and II collagens, which may lead to a subsequent destabilization and further degradation (14). However, these experiments were performed in simple buffer systems. We observed that the addition of GAGs such as C-4S leads to dramatic changes in the collagenolytic activity of cathepsins. None of the tested cathepsins, L, B, S, and F, displayed any detectable collagenase activity even at 37 °C in the presence of C-4S, whereas complete hydrolyses were observed for cathepsins L and S in the absence of C-4S. The inhibition of collagenolysis by C-4S seems to be primarily restricted to the degradation of the triple helical region since the cleavage of telopeptide is still continued, as

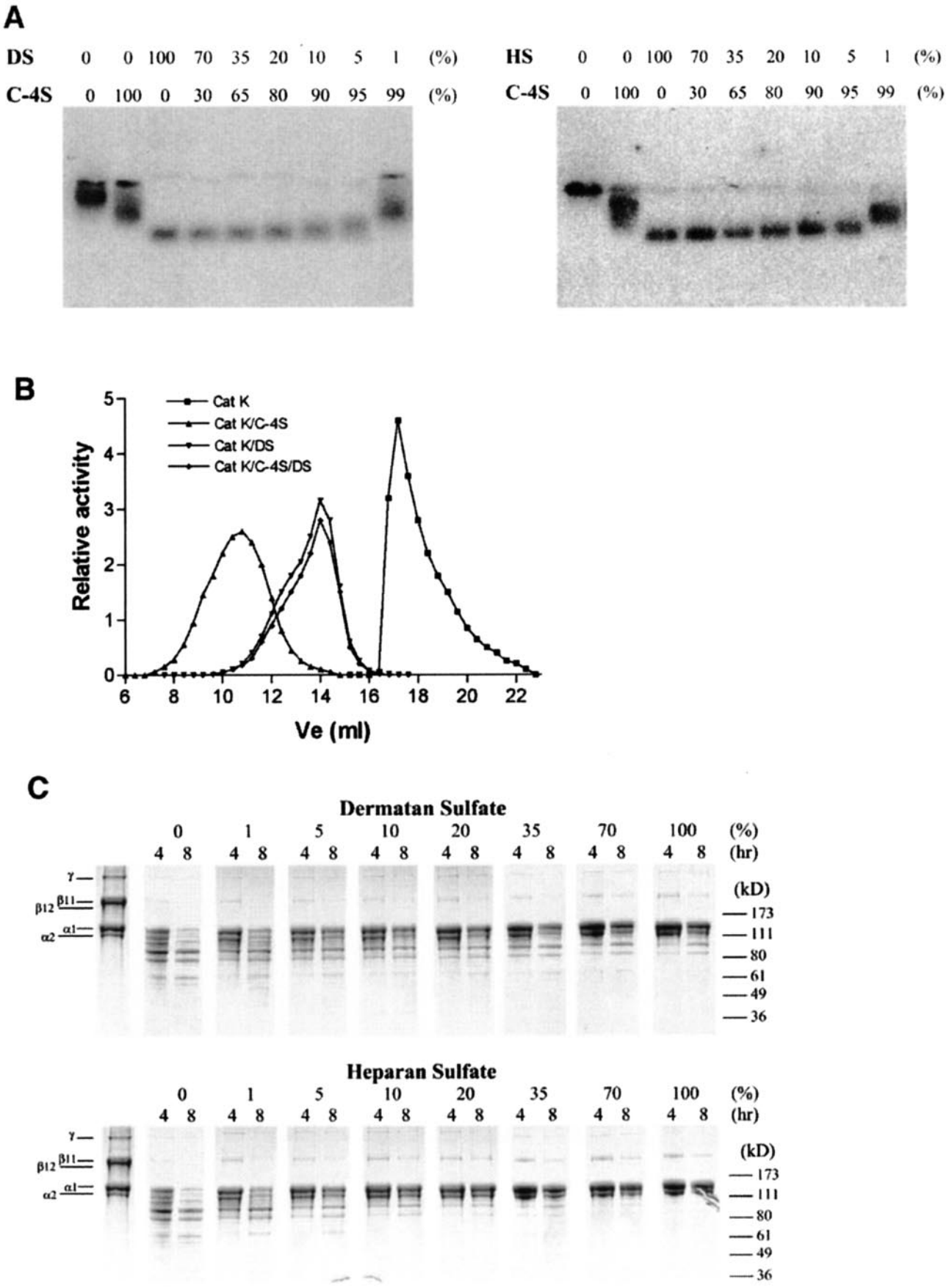
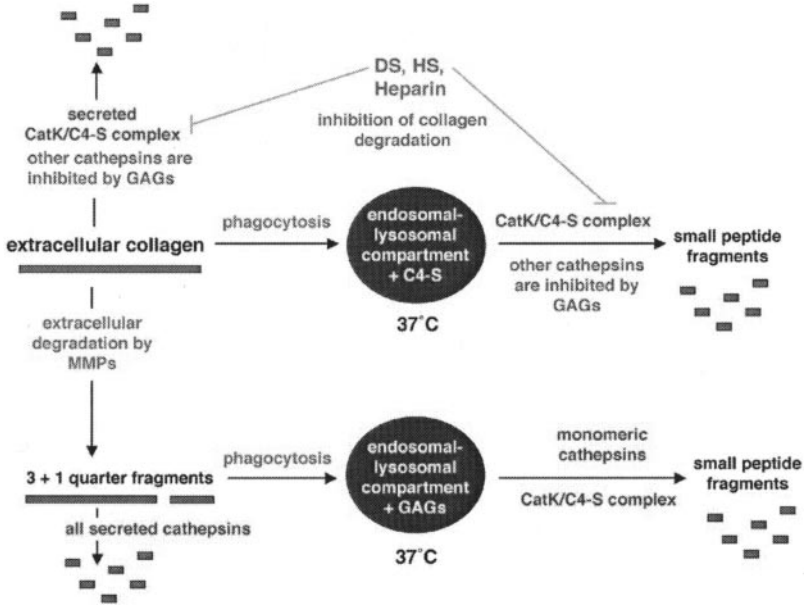


FIG. 10. *A*, electromobility shift assay of ^{125}I-DCG-04-labeled cathepsin K in the presence of increasing amounts of 17-kDa C-4S and decreasing amounts of 30-kDa DS or HS (in %). DS and HS bind more efficiently to cathepsin K and can only be displaced by an excess of C-4S. *B*, gel filtration assay with cathepsin K (Cat K) in the absence and the presence of 30-kDa C-4S and 18-kDa DS using Superdex 200. At equal concentrations (in %) of C-4S and DS, cathepsin K elutes as a DS complex. *C*, displacement of C-4S in cathepsin K-GAG complexes by DS or HS leads to an inhibition of the collagenase activity of cathepsin K. At least a 90% excess of C-4S over DS (in %) and HS is required to allow a significant collagenase activity of cathepsin K. *Ve*, elution volume.



SCHEME 1. **Extra- and intracellular pathways of collagen degradation by cathepsins and MMPs and their regulation by glycosaminoglycans.**

seen by the disappearance of the γ- and β-bands in SDS-polyacrylamide gels. The selective inhibition of cathepsins other than cathepsin K by C-4S may reveal a novel mechanism to control collagen turnover. Collagens are the most abundant extracellular matrix proteins and are responsible for the integrity and structure of most organs and the whole body. An uncontrolled degradation of collagens would be detrimental to life, and, thus control mechanisms must be in place to keep potentially destructive proteases in check. For example, cathepsin L is expressed in most cell types and tissues, and it has also been described that procathepsin L is secreted into the extracellular matrix (41). Released into an acidic extracellular environment, which is typical for many diseased tissues, procathepsin L could be easily auto-activated and would be very effective in destroying collagenous matrices at body temperature. Because endogenous inhibitors of cathepsin L such as cystatin C are down-regulated in diseases as shown in atherosclerosis (42), a second line of defense is necessary against "out of place" collagenolytic cathepsins. Abundantly expressed GAGs such as C-4S can fulfill this task. It should also be considered that extracellular matrix collagen fibril degradation is not necessarily an extracellular degradation process. There is increasing evidence that phagocytosis of extracellular matrix components by fibroblasts and subsequent intracellular hydrolysis contributes significantly to the overall degradation of collagen fibrils (43). The dramatic accumulation of undigested collagen fibrils in human cathepsin K-deficient osteoclasts also challenges the general assumption that osteoclastic collagen degradation primarily happens in the resorption lacuna (25, 44). Also osteoclasts may degrade a significant portion of mostly intact collagen fibrils intracellularly.

Collagenases of the matrix metalloproteinase family (MMP-1, 8, 13) cleave triple helical collagens at a specific single peptide bonds and generate ¼ and ¾ fragments. In general, these fragments were considered as thermally unstable and easily degradable by most other endopeptidases. Our results, however, indicate that MMP-generated fragments show a considerable resistance to further proteolytic degradation when *in vitro* digests were performed in the presence of C-4S at 28 °C. 28 °C is considered a standard temperature for collagen degradation assays since it is below the melting temperature of pepsin-treated solubilized collagens. None of the tested cathepsins (B, L, S, and F) but cathepsin K was able to degrade the MMP-1-generated collagen fragments at 28 °C. In contrast, at 37 °C all cathepsins efficiently degraded the ¾ and ¼ fragments. The apparent melting temperature of soluble triple-helical type I collagen dropped by 8–10 °C when predigested with MMP-1. Thus, the soluble MMP-1-generated collagen fragments are indeed unstable at body temperature and accessible to nonspecific degradation. However, it should be considered that 1) in peripheral joints the body temperature is significantly lower than at core body sites, and thus, the collagen fragments might be resistant to nonspecific degradation, and 2) MMP-nicked collagen molecules cross-linked in the fiber structure might be significantly stabilized. Our qualitative electron microscopic comparison of *in vitro* digested cartilage specimen may support this hypothesis. The most severe changes in the morphology of cartilage matrices were observed after the digestion with cathepsin K. Visible proteoglycan aggregates were completely removed, the type II collagen network of fibrils was apparently collapsed, and individual fiber appeared thinner. Co-digests with MMP-1 and cathepsins L and B could only partially mimic the cathepsin K effect. This may suggest that the MMP-1-generated collagen fragments are not completely accessible to non-collagenolytic cathepsins L and B (cartilage contains high amounts of C-4S, inhibiting the collagenolytic activities of cathepsins other than cathepsin K).

Scheme 1 summarizes the potential pathways for the intra- and extracellular type I and II collagen turnover by cathepsins and matrix metalloproteinases. Extracellular fibrillar collagens are degraded by MMPs by generating ¼ and ¾ cleavage fragments. These collagen fragments are thermally unstable at 37 °C and can be easily degraded by any other secreted cathepsin provided that there is an acidic environment. The presence of GAGs does not interfere with the cathepsin-mediated degradation of MMP-generated collagen fragments. Thus, in this pathway the MMPs represent the rate-limiting activities. On the other hand, intact collagen fibrils in the extracellular matrix can be directly degraded by cathepsin K in the presence of "productive" GAGs. Cathepsin K in the presence of C-4S, C-6S, and KS forms collagenolytically active complexes that can efficiently hydrolyze collagen fibrils in an acidic microenvironment. However, the presence of sufficient concentrations of competing "non-productive" GAGs such as DS, HS, and/or heparin can inhibit active cathepsin K complexes by forming alternative, collagenolytically inactive cathepsin K complexes. The extracellular pathway of collagen turnover is complemented by an appropriate intracellular pathway (43). Again,

MMP-predigested collagen taken up by cells is easily degraded by all lysosomal cathepsins irrespective of the presence of GAGs. However, phagocytosed collagen fibrils are likely to be degraded exclusively by cathepsin K complexes since C-4S and other GAGs will inhibit other cathepsins even at body temperature. Similar to the extracellular pathway, non-productive GAGs can regulate the activity of cathepsin K within the lysosomal-endosomal compartment.

The inhibition of cathepsin K complexes by DS, HS, and heparin might be highly relevant for the regulation of collagen turnover during health and disease. It has been shown that the ratios of GAGS within tissues undergo significant changes during development and aging (45–49). For example, heparanase, which primarily hydrolyzes HS but also C-4S, is highly expressed in various tumor cells and strongly facilitates tumor invasion and tumor angiogenesis (50). It is conceivable that the hydrolysis of HS leads either to an increase of cathepsin K/C-4S complexes or, if all GAGs are depleted, to the activation of the collagenolytic activity of cathepsin L and, thus in either case, to an increased degradation of the extracellular matrix around the acidic perimeter of tumors, leading to an enhancement of tumor invasiveness.

In summary, there are two potential pathways of collagen catabolism. First, the MMP/cathepsin pathway depends on the rate-limiting activities of MMP-collagenases and the subsequent degradation of thermal unstable collagen fragment by many other proteases including cathepsins and non-collagenolytic MMPs. This pathway can be regulated at the transcriptional and/or translational level or by endogenous MMP inhibitors such as tissue inhibitor of metalloproteinases. On the other hand, the cathepsin pathway can, in addition to transcriptional/translational and endodogenous inhibitor mechanisms, be effectively controlled by GAGs. The cathepsin-mediated degradation route does not need the cooperation of other protease classes. On one side, productive GAGs render monomeric cathepsin K into an extremely potent mammalian collagenase, and non-productive GAGs are able to silence this activity. Moreover, most if not all GAGs inhibit other collagenolytically active cysteine proteases such as cathepsin L and make collagen a specific substrate for cathepsin K in a physiologically relevant environment. The fact that the cathepsin K mutant, Y212C, is unable to form complexes with GAGs and, thus, specifically lost its collagenase activity (28) and causes the bone-sclerosing disorder, pycnodysostosis (22), suggests that the cathepsin K-GAG complex pathway represents the major and critical mechanism of bulk collagen turnover.

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