Collagenolytic Activity of Cathepsin K Is Specifically Modulated by Cartilage-Resident Chondroitin Sulfates†

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ABSTRACT: Cathepsin K is the predominant cysteine protease in osteoclast-mediated bone remodeling, and the protease is thought to be involved in the pathogenesis of diseases with excessive bone and cartilage resorption. Osteoclastic matrix degradation occurs in the extracellular resorption lacuna and upon phagocytosis within the cell's lysosomal-endosomal compartment. Since glycosaminoglycans (GAGs) are abundant in extracellular matrixes of cartilage and growing bone, we have analyzed the effect of GAGs on the activity of bone and cartilage-resident cathepsins K and L and MMP-1. GAGs, in particular chondroitin sulfates, specifically and selectively increased the stability of cathepsin K but had no effect on cathepsin L and MMP-1. GAGs strongly enhanced the stability and, to a lesser extent, the catalytic activity of cathepsin K. To combine the activity and stability parameters, we defined a novel kinetic term, named cumulative activity (CA), which reflects the total substrate turnover during the life span of the enzyme. In the presence of chondroitin-4-sulfate (C-4S), the CA value increased 200-fold for cathepsin K but only 25-fold with chondroitin-6-sulfate (C-6S). C-4S dramatically increased the hydrolysis of soluble as well insoluble type I and II collagens, whereas the effects of C-6S and hyaluronic acid were less pronounced. C-4S acts in a concentration-dependent manner but reaches saturation at approximately 0.1%, a concentration similar to that found in the synovial fluid of arthritis patients. C-4S increased the cathepsin K-mediated release of hydroxyproline from insoluble type I collagen 10-fold but had only a less than 2-fold enhancing effect on the hydrolysis of intact cartilage. The relatively small increase in the hydrolysis of cartilage by C-4S was attributed to the endogenous chondroitin sulfate content present in the cartilage. Although C-4S increased the pH stability at neutral pH, a significant increase in the collagenolytic activity of cathepsin K at this pH was not observed, thus suggesting that the unique collagenolytic activity of cathepsin K at acidic pH is mechanistically determined and not by the enzyme's instability at neutral pH. The selective and significant stabilization and activation of cathepsin K activity by C-4S may provide a rationale for a novel mechanism to regulate the enzyme's activity during bone growth and aging, two processes known for significant changes in the GAG content.

The organic matrix of cartilage is composed of type II collagen and various proteoglycans. The largest in size and most abundant proteoglycan is aggrecan consisting of a three globular domain protein backbone (∼260 kDa) with over 100 O-linked chondroitin and keratan sulfate side chains (each 20−30 kDa). Aggrecan molecules interact with hyaluronic acid to form large aggregates (*1*). The hydrophilic character of the polysaccharide component in these aggregates gives the articular cartilage its osmotic properties which are responsible for the resistance against compressive loads. In addition to covalently bound polysaccharides, soluble chondroitin sulfates are found in the cartilage and synovial fluid of the joint capsule. Shinmei et al. (*2*) described high concentrations of chondroitin disaccharides after chondroitinase ABC digestion in pathologic joints reaching 55 μM in rheumatoid arthritis and 211 μM in traumatic arthritis. Several lines of evidence indicate that type I and II collagen and aggrecan degradation is mediated by matrix metalloproteinases and cysteine proteases and that osteo/chondroclasts, chondrocytes, and synovial fibroblasts are the major sources of these activities. We and others have identified cathepsin K as the predominant cysteine protease in osteoclasts (*3*−*5*) and most recently also in synoviocytes of RA patients (*6*). Furthermore, we and others have shown that cathepsin K is an efficient and unique collagenase cleaving type I and II collagens in their helical domains (*7*, *8*). The critical involvement of cathepsin K in osteoclastic bone resorption was eventually confirmed by the finding that deficiency in cathepsin K activity causes a rare bone-sclerosing disorder named pycnodysostosis (*9*) and that disease-related mutations in the cathepsin K gene lead to the loss of its collagenolytic activity (*10*). The presence of cathepsin K in chondroclasts and RA-synoviocytes suggests that the protease is involved in the normal and pathological turnover of cartilage. In this report, we demonstrate that cathepsin K activity is specifically modulated by chondroitin sulfates present in cartilage.

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EXPERIMENTAL PROCEDURES

Materials. *Z*-Leu-Arg-MCA[1] was synthesized as described (*11*). Chondroitin 4-sulfate (C-4S) chondroitin 6-sulfate (C-6S), dermatan sulfate (DS), and hyaluronic acid (HA) were purchased from Sigma (St. Louis, MO).

Proteases. Human cathepsin K was expressed in *P. pastoris* and purified by ion-exchange chromatography using SP Sepharose (Pharmacia-Upjohn) as described (*12*). Human cathepsin L also was produced in *P. pastoris* (unpublished results, DB). Activated recombinant human MMP-1 was a generous gift from H. Nagase (University of Kansas Medical Center, Kansas City, KS). Molar concentrations of cathepsins K and L were determined by active site titration using E−64 (*13*).

Immunolocalization of Cathepsin K in Chondroclasts. Archived paraffin-embedded rheumatoid arthritis specimens were kindly provided by Dr. M. Klein (Department of Pathology, Mount Sinai School of Medicine, New York). Paraffin sections (6 μM) were mounted onto Vectabond slides (Vector Laboratories, CA), dried overnight, and dewaxed with xylene. Immunostaining was performed using the StrAviGen Super Sensitive Immunodetection B-SA System (Biogenix, San Ramon, CA) and monoclonal anti-cathepsin K (M21, kindly provided by Justine Whaley NovaDx, San Diego; 1:200 dilution). Counterstaining was performed with Meyer's hematoxylin and eosin. The slides were evaluated using a departmental Nikon Eclipse E800 microscope. Nonspecific mouse IgG (Biogenix) was used as control for the primary antibody.

Cathepsin Assay with Methylcoumarylamide Substrate. Initial rates of methylcoumarylamide substrate hydrolysis were monitored in 1 cm cuvettes at 25 °C in a Perkin-Elmer fluorimeter at excitation and emission wavelengths of 380 and 450 nm, respectively. Recombinant human cathepsin K was assayed at constant enzyme concentration (0.3−1 nM) and variable substrate concentrations in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM dithiothreitol and 2.5 mM Na_2EDTA. The kinetic parameters were determined in the presence or the absence of 0.15% (w/v) polysaccharides (C-4S, C-6S, DS, and HA) or 0.15% bovine aggrecan. It was estimated that the ratio of protein to GAG is 1:12.5 in aggrecan. The aggrecan protein concentration was determined using the Bio-Rad protein assay. In experiments with GAGs, the cathepsin stock solutions were prepared by preincubating enzymes with GAGs for 20 min. The kinetic constants V_{max} and K_m were obtained by nonlinear regression analysis using the program Enzfitter (*14*). Active-site concentration of cathepsins K and L was determined with E−64 titration (*13*).

Cumulative Activity (CA). CA reflects the total activity of an enzyme in its life span and, thus, considers both reaction velocity and enzyme stability (eq 1):

$$CA = AT/E \quad (1)$$

where T is the cumulative lifetime of a defined enzyme quantity(s), A is the enzyme activity (mol/s), and E is the enzyme amount (mol). T is equal to the area beneath a stability time course. Under the condition of substrate saturation, CA corresponds to CA_{max} and can be calculated by eq 2:

$$CA_{max} = k_{cat}T \quad (2)$$

CA has no dimension but reflects moles of substrate hydrolyzed per mole of enzyme during its average catalytic lifetime. We determined the CA values of cathepsins K and L for the substrate *Z*-Leu-Arg-MCA at pH 5.5, 28 °C, in the presence and the absence of 0.15% of GAGs. T values were obtained using the NIH image program.

Preparation of Bovine Aggrecan. Proteoglycan aggregates were extracted from bovine neck cartilage in 4 M guanidinium chloride in 50 mM sodium acetate buffer, pH 5.8, containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μM E-64, and 1 μg/mL pepstatin. The aggregates were purified in a cesium chloride gradient as described by Heinegard (*15*). The gradient was cut in three fractions, A1 to A3, with the proteoglycans present in the bottom fraction, A1. Aggrecan aggregates were dialyzed against water at 4 °C for 24 h.

pH Activity Profiles and pH Stability. The pH activity profile of human cathepsin K was determined at 1.5 μM substrate (*Z*-Leu-Arg-MCA) concentration ([S] < K_m where the initial rate, v_o, is directly proportional to the k_{cat}/K_m value). The following buffers were used for the pH activity profile: 100 mM sodium citrate (pH 2.8−5.8), 100 mM sodium phosphate (pH 5.8−8.0), and sodium borate (pH 8.0−10.0). All buffers contained 1 mM EDTA, 2.5 DTT, and 0.4 M NaCl to minimize the variation in ionic strength. A three protonation model was used for least-squares regression analysis of the pH activity data (*16*). The data were fitted to the following equation:

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)/([H^+]/K_1 + 1 + K_2/[H^+]) \quad (3)$$

pH-dependent stability of active cathepsins K and L was determined in 100 mM sodium acetate buffer, pH 5.5, and in potassium phosphate buffer, pH 6.5, 7.0, and 7.5. At appropriate time intervals, aliquots of the incubation mixture were withdrawn and the activity was measured using the fluorogenic substrate assay described above. pH activity profiles and pH stability was measured in the absence and presence of 0.15% C-4S.

Collagen Digests. Collagen type I (0.4 mg/mL) (calf skin, Calbiochem) or collagen type II (0.4 mg/mL) (calf articular joints/Amersham) were incubated with human cathepsin K (800 nM) or human cathepsin L (400 nM) in sodium acetate buffer, pH 5.5, containing 2.5 mM dithiothreitol and EDTA or with MMP-1 (250 nM) in 100 mM Tris/HCl buffer, pH 7.2, and 10 mM $CaCl_2$. Collagen digestion was performed at 28 °C in the absence and the presence of 0.15% (w/v) GAGs. The digestion reaction was stopped by the addition of 10 μM E-64 for cathepsins K and L, 20 mM EDTA for MMP-1. The samples were subjected to SDS−polyacrylamide electrophoresis using 4−20% Tris/glycine gels (Novex), and the gels were stained with Coomassie Blue.

Degradation of Insoluble Type I Collagen and Cartilage. Insoluble bovine collagen of type I (3.5 mg/mL) (achilles

[1] Abbreviations: Z-, benzyloxycarbonyl; -MCA, 4-methyl-7-coumarylamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; DTT, dithiothreitol; MMP, matrix metalloproteinase; GAG, glycosaminoglycan; C-4S, chondroitin-4-sulfate, C-6S, chondroitin-6-sulfate; HA, hyaluronic acid; DS, dermatan sulfate; CA, cumulative activity. The triple letter code for amino acids was used.

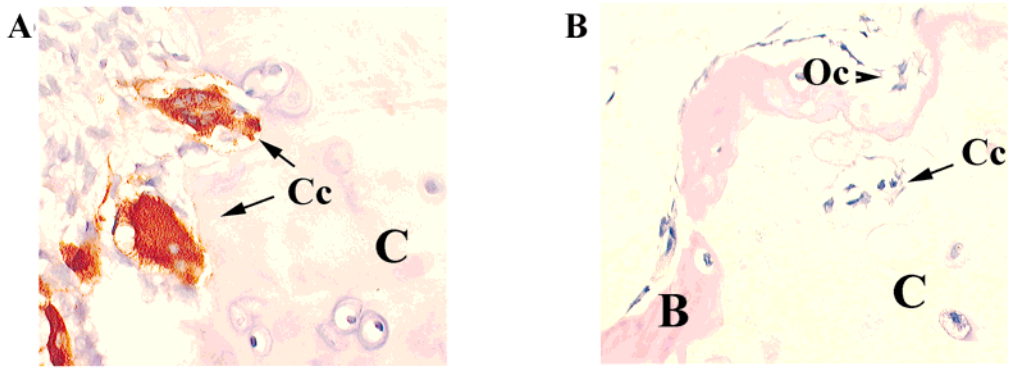

FIGURE 1: Immunohistochemical staining of cathepsin K expression in chondroclasts. (A) Chondroclasts in contact with articular cartilage from a rheumatoid arthritis specimen show expression of cathepsin K. The arrowheads indicate two chondroclast facing the cartilage surface. Cells were stained with M21, a monoclonal anti-human cathepsin K antibody. (B) Chondroclasts stained with control mouse IgG. Counterstaining with hematoxylin and eosin. C, cartilage; B, bone; Cc, chondroclast; Oc, osteoclast.

tendon, Sigma) and 45 mg of finely minced bovine cartilage pieces (approximately 1 mg) (Animal Organ and Tissues for Research, NJ) were washed three time with 100 mM acetate buffer, pH 5.5, and incubated with 800 nM recombinant cathepsin K in 900 μL of 100 mM sodium acetate buffer, pH 5.5, in the absence or the presence of 0.15% C-4S at 28 °C or with 400 nM MMP-1 at pH 7.2 (100 mM Tris/HCl containing 10 mM $CaCl_2$). A total of 100 μL of supernatant was taken after 24 h and hydrolyzed in 6 M HCl for 18 h at 105 °C in a sealed hydrolysis vial. The hydroxyproline content from soluble collagen fragments was measured according to the method described by Firschein and Shill (*17*). Briefly, hydrolysis samples were neutralized at 4 °C by NaOH in 0.25 M sodium citrate buffer containing 1 M acetate, pH 6.0. A total of 100 μL of fresh chloramine-T-reagent containing 1.41% (w/v) chloramine T, 20% (v/v) H_2O, 30% (v/v) ethylene glycol monomethyl ether (EGME), and 50% (v/v) citrate buffer described above were added to 200 μL of the sample, incubated for 20 min and supplemented with 100 μL of 6.3 M perchloric acid. After 5 min, 100 μL of 20% w/v fresh *p*-dimethylaminobenzaldehyde in EGME was added. The color was developed at 60 °C for 20 min. After cooling to room temperature, optical densities were read at 546 nm. Hydroxyproline content from soluble collagen fragments was determined using hydroxyproline (Sigma) as standard.

RESULTS

Localization of Cathepsin K in Chondroclasts. M21, monoclonal anti-human cathepsin K antibody, stained specifically multinucleated osteo- and chondroclasts in tissue sections of an rheumatoid arthritis specimen. Strong vacuolar staining was found in multinucleated cells attached to articular cartilage which were considered as actively resorbing chondroclasts (*18*). Figure 1 shows two multinucleated chondroclasts invading cartilage matrix and coming in close contact with cartilage-embedded chondrocytes. The formation of deep Howship's lacunae indicates that the final breakdown and removal of cartilage tissue is mediated by chondroclasts.

Substrates for the chondroclasts are primarily type II collagen and aggrecan. Aggrecan is the most abundant proteoglycan in cartilage. Due to its numerous O-linked sulfated polysaccharides, it forms a strongly hydrophilic and negatively charged matrix. Therefore, proteolytic activities contributing to cartilage breakdown will encounter a microenvironment where sulfated GAGs are abundant. Thus, we determined the effects of various GAGs on the activities of three collagenolytic proteases, cathepsins K, cathepsin L, and MMP-1.

Stability and Activity of Cathepsins K and L in the Presence of Chondroitin Sulfates, HA, and Aggrecan. We first investigated whether cartilage-resident GAGs are able to stabilize the activity of cathepsins K and L. C-4S, C-6S, DS, and HA at concentrations of 0.15% were individually incubated with cathepsins K and L at 28 °C and pH 5.5 for 2 h and residual activities of the proteases were determined. The assay concentration of 0.15% for GAGs was chosen since this is in the range of chondroitin sulfate concentrations (0.1−0.2%) determined in the synovial fluid of RA, OA, and traumatic arthritis patients (*2*). Table 1A shows that GAGs significantly and specifically stabilize cathepsin K activity whereas no effect was observed on the stability of cathepsin L. Among the tested GAGs, C-4S proved as most effective. The residual activity of cathepsin K after 2 h increased from 5 to 98% at pH 5.5. Table 1B displays the pH dependence of cathepsins K and L in the presence of C-4S. The residual activity of cathepsin K increased from 15 to 100% at pH 5.5 and from 5.4 to 42.1% at pH 7.0 in the presence of C-4S. As expected C-4S did not effect the pH stability of cathepsin L. Next, we determined the concentration dependency of C-4S on the stability of cathepsins K and L. Maximal stabilization of cathepsin K activity was achieved at C-4S concentrations above 0.1%. The activity profile follows saturation kinetics, and no further increase was observed up to 0.3% C-4S (Figure 2). In contrast, no stabilization of cathepsin L was observed in the concentration range tested.

To determine whether the increase of residual activity of cathepsin K in the presence of GAGs is due to an increase in the enzyme's stability or its catalytic activity, the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined. C-4S, C-6S, and DS increased the k_{cat}/K_m value for the cathepsin K-catalyzed hydrolysis of *Z*-Leu-Arg-MCA by a factor of 2 (Table 2). This increase is mostly due to an increase in the k_{cat} value. HA does not have an effect on cathepsin K activity. As expected, the catalytic activity of cathepsin L is affected by none of the tested GAGs. Since chondroitin sulfates increased both the stability and the activity of cathepsin K, we defined a novel kinetic parameter, named cumulative

Table 1: Stability of Recombinant Human Cathepsins K and L in the Presence and the Absence of Glycosaminoglycans

(A) Residual Activities of Human Recombinant Cathepsins K and L after 2 h Incubation at pH 5.5 and 28 °C in the Absence or the Presence of 0.15% Glycosaminoglycans[a]

protease	no GAG	C-4S	DS	C-6S	HA
cathepsin K	5.3 ± 0.9	97.8 ± 13.9	78.0 ± 8.0	53.2 ± 9.9	43.6 ± 6.3
cathepsin L	91.9 ± 6.0	92.4 ± 6.7	94.6 ± 5.2	93.7 ± 5.8	93.3 ± 9.1

(B) Residual Activities of Human Recombinant Cathepsins K and L after 1 h Incubation at 28 °C and at Different pH Values in the Absence or the Presence of 0.15% C-4S[a]

protease	GAG	pH 5.5	pH 6.5	pH 7.0	pH 7.5
cathepsin K	no C-4S	14.9 ± 1.9	13.3 ± 5.5	5.4 ± 0.9	3.6 ± 1.6
	+C-4S	102.1 ± 9.3	76.3 ± 5.1	42.1 ± 1.8	13.2 ± 0.4
cathepsin L	no C-4S	95.2 ± 3.5	34.4 ± 3.0	0	0
	+C-4S	96.0 ± 4.1	37.3 ± 5.5	0	0

[a] The data represent the percentage of residual activity as mean ± S.D values of two to four experiments. The residual activity was measured using *Z*-Leu-Arg-MCA as substrate.

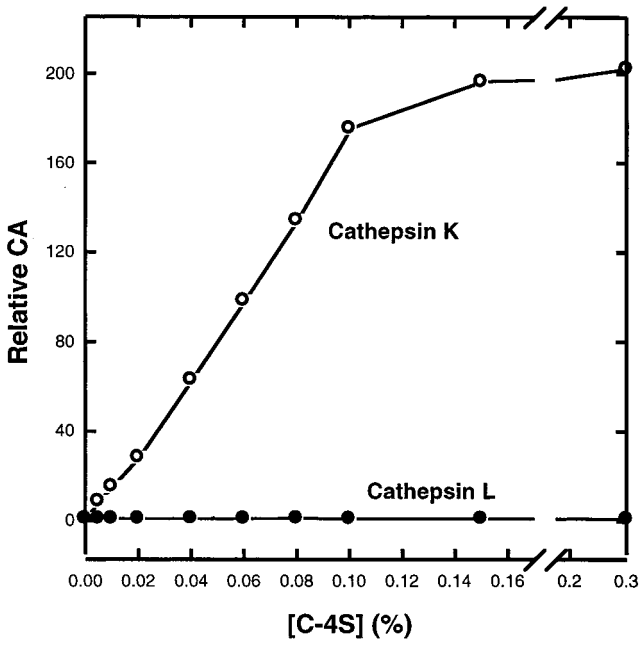

FIGURE 2: Chondroitin-4-sulfate dependence of human cathepsins K and L. Cumulative activity of cathepsins K and L were determined as described in the Experimental Procedures. The experiments were performed at pH 5.5, 28 °C, 5 μM *Z*-Leu-Arg-MCA with various concentrations of C-4S ranged 0−0.3%. The CA values in the absence of C-4S were normalized as 1 (5.029×10^3 mol of substrate/mol of enzyme for cathepsin K; 1.872×10^6 mol of substrate/mol of enzyme for cathepsin L).

activity (CA). CA reflects the total activity of an enzyme in its life span and, thus, considers both reaction velocity and enzyme stability (see the Experimental Procedures). This parameter is useful to quantify the effects of modulators on enzyme stability and activity. We determined the CA values for cathepsins K and L in the presence of different GAGs. As shown in Figure 3, chondroitin 4-sulfate increases the CA value approximately 200-fold, i.e., each cathepsin K molecule is able to hydrolyze 200 times more substrate molecules during its catalytic lifetime in the presence of chondroitin 4-sulfate than in its absence. Interestingly, the CA value for C-6S was significantly lower than that determined for C-4S (total increase in activity = 25-fold), which suggests that different GAGs interact specifically with cathepsin K. Bovine aggrecan increased the CA value by 14-fold, indicating that also O-linked glycosaminoglycans significantly stabilize cathepsin K activity. The lowest increase in CA was observed for HA (10-fold). In contrast, there was no significant effect on the CA of cathepsin L for all GAGs tested.

Effect of GAGs on the Activity of Cathepsin K, Cathepsin L, and MMP-1 on the Cleavage of Type I and II Collagens. Type I and II collagens were incubated in the presence and absence of various cartilage-resident polysaccharides with cathepsins K and L at pH 5.5 and with MMP-1 at pH 7.2. The degradation of both types of collagens is strongly enhanced by C-4S (Figure 4), less by C-6S, but not by DS and HA (not shown for C-6S and DS in Figure 5). In the absence of stabilizing GAGs, various high molecular cleavage products of collagens I and II were observed after 12 h of incubation with cathepsin K, which disappeared in the presence of 0.15% chondroitin sulfates. Increased hydrolysis rates for type II collagen also were observed in the presence of bovine aggrecan, indicating that O-linked GAGs are similarly effective as free GAGs (Figure 4B). Furthermore, the chondroitin sulfates appear to alter the cleavage specificity of cathepsin K as documented by the different cleavage pattern**.** However, this also may be caused by an increased susceptibility of certain collagen fragments to cathepsin K-mediated hydrolysis by the added polysaccharides. Interestingly, none of the GAGs caused an increase in type I or II collagen degradation by cathepsin L and MMP-1, thus indicating specific interactions of the GAGs with cathepsin K (Figure 5).

To address the effect of C-4S on the hydrolysis of insoluble and cross-linked collagens, we incubated insoluble bovine type I collagen and bovine cartilage pieces with recombinant cathepsins K or L and measured the release of soluble collagen fragments by determining the hydroxyproline (Hyp) content. As for soluble type I collagen, the hydrolysis rate for insoluble collagen was dramatically increased in the presence of C-4S for cathepsin K. Hyp concentrations measured in the solubilized collagen fraction increased 10-fold (Figure 6). The increase for that of type II collagen containing cartilage was less significant. However, since cartilage was used as substrate, it is likely that the endogenous content of GAGs within the cartilage is already sufficient for the stabilization of cathepsin K and that their concentration is in the range where we have observed saturation by C-4S (see Figure 2). In contrast, MMP1 activity toward insoluble type I collagen and cart-

Table 2: Kinetic Parameters for the Hydrolysis of Z-Leu-Arg-MCA by Recombinant Human Cathepsins K and L in the Presence of Glycosaminoglycans at pH 5.5

	cathepsin K			cathepsin L		
GAGs	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^{6M-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^6\ M^{-1}\ s^{-1}$)
0% GAGs	4.4 ± 0.1	4.0 ± 02	1.1	14 ± 1	8 ± 0.7	1.75
0.15% C-4S	11 ± 0.2	5.4 ± 0.2	2.04	17 ± 1	10.9 ± 0.9	1.56
0.15% C-6S	10 ± 0.3	4.7 ± 0.3	2.13	17 ± 1	9.2 ± 0.9	1.85
0.15% DS	9 ± 0.3	4.2 ± 0.3	2.14	18 ± 1	10.4 ± 0.6	1.73
0.15% HA	4.2 ± 0.2	3.8 ± 0.4	1.1	16 ± 1	8.8 ± 0.8	1.82
0.15% aggrecan	6.5 ± 0.2	6.5 ± 0.5	1.0	nd	nd	nd

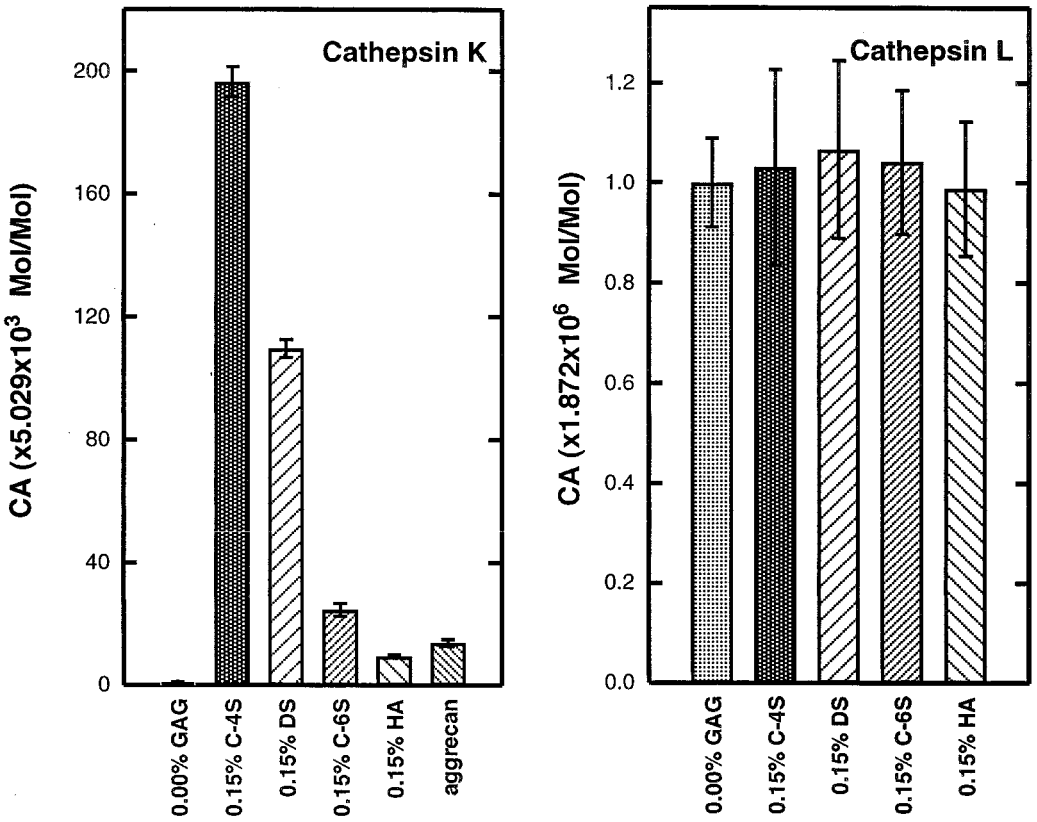

FIGURE 3: Effects of different glycosaminoglycans on cumulative activity of human cathepsins K and L. Cumulative activity of cathepsins K and L were determined by the method described in the Materials and Methods. The experiments were performed at pH 5.5, 28 °C, 5 μM Z-Leu-Arg-MCA in the absence or the presence of 0.15% of free GAGs (C-4S, chondroitin-4-sulfate; C-6S, chondroitin-6-sulfate; DS, dermatan sulfate; HA, hyaluronic acid) or 0.15% GAGs in bovine aggrecan. The CA values in the absence of GAG were normalized as 1 (5.029×10^3 mol of substrate/mol of enzyme for cathepsin K; 1.872×10^6 mol of substrate/mol of enzyme for cathepsin L). All CA values represent the mean ± SD of four experiments.

ilage was very low and not influenced by C-4S.

Effect of C-4S on pH Stability, pH Activity Profile, and pH Dependence. C-4S revealed the strongest effect on the CA of cathepsin K. This was primarily due to the increase in the enzyme's stability. To examine the effect of C-4S on the pH stability of cathepsins K and L, we determined the residual activity of both cathepsins in the presence and absence of C-4S in the pH range of 5.5 to 7.5. C-4S increased the residual activity of cathepsin K about 5−10-fold over the range tested, when compared with the activity measured in the absence of the polysaccharide (Table 1). The half-life of cathepsin K increased at pH 7.0 from 6 to 40 min. No stabilization of cathepsin L activity was observed. The analysis of the pH activity profiles of cathepsin K revealed a broadened pH-bell curve with an increase of the width of the profile by 0.3 pH units (Table 3). The width of pH-activity profiles of papain-like cysteineproteases is regarded as a measure of the ion-pair stability of the catalytic cysteine and histidine residues. Thus, the significant increase of the pH-profile width is interpreted as an increase of the intrinsic stability of cathepsin K (*19*).

Type I and II collagens were incubated with cathepsin K for 12 h in the absence and presence of C-4S at pH values between 4 and 7, and collagen hydrolysis was followed by SDS−PAGE. The efficiency of cathepsin K to hydrolyze both substrates increased over the whole pH range but the presence of C-4S did not alter the pH optimum of the protease. However, despite the increased pH stability of cathepsin K at neutral pH, there was no significant increase in collagen hydrolysis observed (Figure 7).This suggests that the collagenolytic activity of cathepsin K requires an acidic pH, whereas synthetic substrates and noncollagenous proteins are accessible to degradation by cathepsin K also at neutral pH.

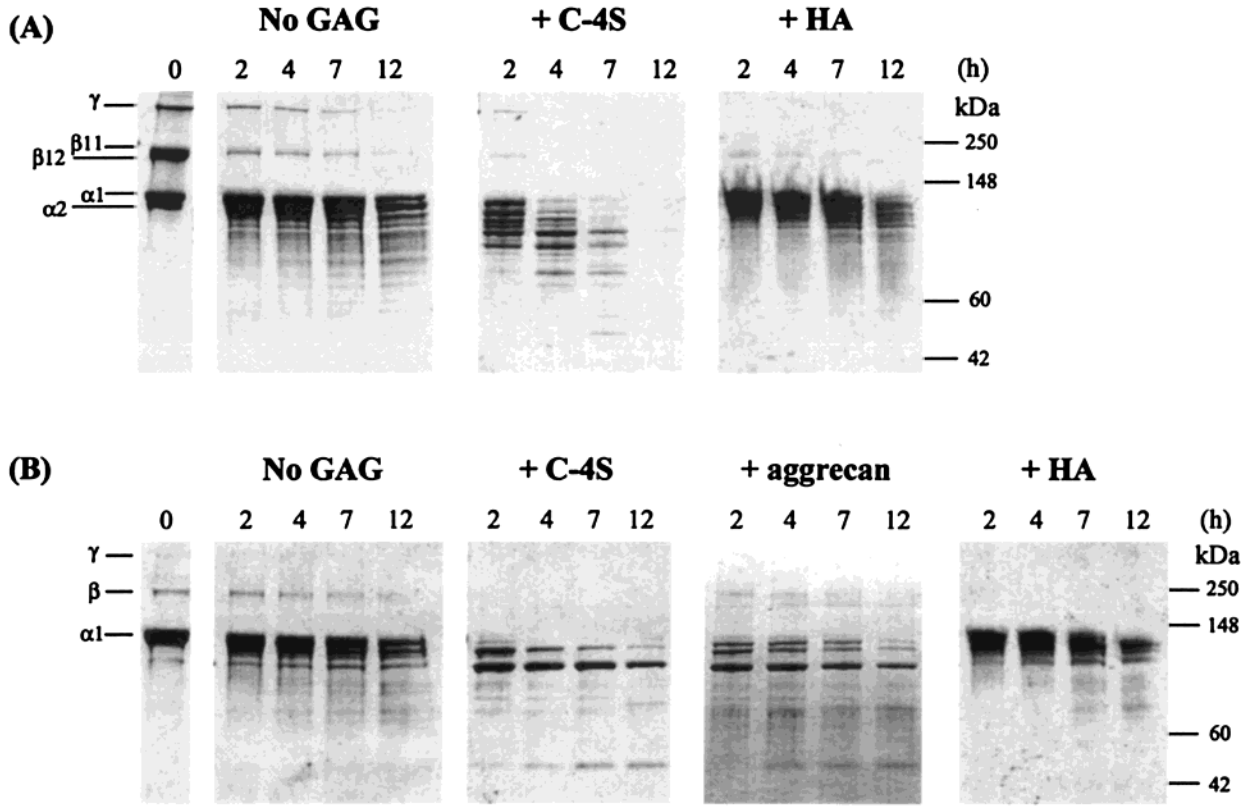

FIGURE 4: Degradation of soluble types I and II bovine collagen by recombinant human cathepsin K in the absence and the presence of C-4S and HA. (A) Soluble type I or (B) type II bovine collagens were digested with 800 nM cathepsin K at 28 °C, pH 5.5, in the absence or the presence of 0.15% C-4S or 0.15% HA. In addition, type II collagen was digested in the presence of aggrecan containing an equivalent of 0.15% GAGs. The reaction was stopped by the addition of 5 μM E-64 after various time periods (shown in the top margin) and the samples were analyzed by 4−20% SDS−PAGE. Molecular mass standards are indicated in the right lane.

DISCUSSION

Matrix metalloproteinases and papain-like cathepsins are known as potent extracellular matrix-degrading hydrolases in bone and cartilage remodeling. Both protease classes encounter a complex extracellular and/or intralysosomal microenvironment which is characterized by high concentrations of GAGs. However, most in vitro experiments are performed in simple buffer systems which do not reflect the in vivo conditions of an extracellular or lysosomal fluid. The data presented in this study demonstrate that GAGs selectively modulate the activity of cathepsin K whereas no effects on the activities of cathepsin L and MMP were observed. Cathepsin K is characterized by its unique collagenase activity and predominant expression in osteoclasts and thus thought to be one of the most important proteolytic activities in osteoclastic bone and cartilage resorption. The biological role of this papain-like cysteine protease in bone digestion was confirmed by the finding that cathepsin K-deficiency causes a rare osteosclerotic bone disease, pycnodysostosis, which, on a cellular level, is distinguished by large demineralized areas adjacent to osteoclasts and an accumulation of undigested collagen fibrils in the vesicular compartment of these cells (*9*, *20*). Our findings that GAGs selectively and specifically increase the cathepsin K potential to hydrolyze collagens further support the specific function of this protease in osteoclast-mediated bone and cartilage digestion and suggest a novel mechanism of cathepsin regulation. The increase in total activity is primarily based on an increase in the enzyme's lifetime and pH stability. The stabilization is dependent on the structure of the GAGs and dramatically varies with even small changes in their structures. C-4S and C-6S, which are distinguished only by their sulfatation at the C-4 and C-6 position of *N*-acetyl-β-galactosamine, reveal an almost 10-fold difference in their stabilizing effect on cathepsin K. Considering tissue-specific and age-related changes in the GAG content, those differences may directly influence cathepsin K activity. For example, the absolute concentration of C-4S is three times higher at the endochondral ossification zone when compared to the articular surface (*21*). However, with age and at tissue sites where the ossification is complete, the C-4S content decreases whereas the relative content of C-6S increases (*22*). The approximately 200-times higher CA of cathepsin K in the presence of C-4S when compared with C-6S suggests that the higher C-4S concentration in developing endochondral bone increases site-specific cathepsin K-mediated bone resorption during the bone growth. This is supported by our finding that cathepsin K degraded type I collagen more efficiently in the presence of C-4S than C-6S.

Osteo/chondroclasts which actively resorb cartilage have to degrade type II collagen in the presence of abundant aggrecan or their degradation products. We have previously demonstrated that cathepsin K is able to hydrolyze native type II collagen in its helical region (*7*). Here, we could show that the degradation of articular collagen is greatly enhanced in the presence of aggrecan or C-4S and that the increase in hydrolysis efficiency is specific for cathepsin K since no change was observed with regard to the cathepsin L and MMP-1 activities.

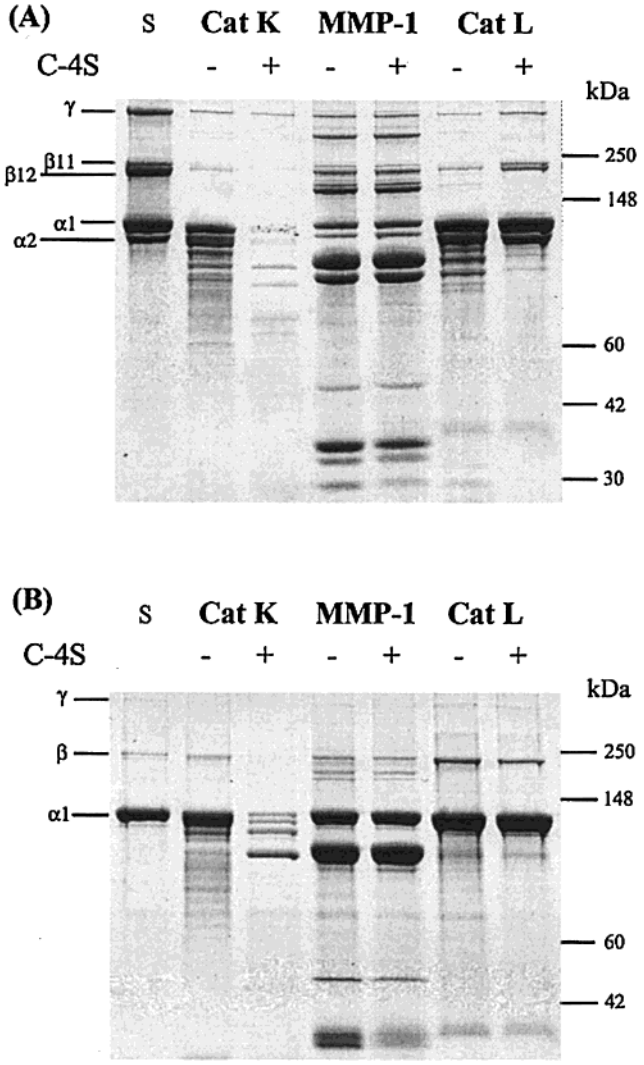

FIGURE 5: Comparison of collagenolytic effects of C-4S on cathepsins K and L and MMP-1. (A) Soluble type I or (B) type II bovine collagens were incubated in the absence or the presence of 0.15% C-4S at 28 °C with 400 nM cathepsin K or L at pH 5.5 for 8 h or with 240 nM MMP-1 at pH 7.2 for 24 h. The samples were analyzed by 4−20% SDS−PAGE. Untreated soluble collagen was used as standard (S). Molecular mass standards are indicated in the right lane.

It is of interest that chondroitin sulfates significantly increase the pH stability of cathepsin K at neutral pH but that this increase in stability does not translate into an increase in degradation of type I and II collagens at pH values above pH 6.5. This indicates that cathepsin K-mediated collagen hydrolysis is restricted to an acidic pH and thus confined to the subosteoclastic and lysosomal compartments. However, noncollagenous proteins such as aggrecan display significant hydrolysis at neutral pH by cathepsin K (D.B., unpublished data), suggesting a considerable activity of the protease in an extracellular environment saturated with GAGs. Here, it is of particular interest that cathepsin K has been described to be expressed in synovial fibroblasts of rheumatoid arthritis (RA) patients (*6*). RA is characterized by an irreversible and excessive destruction of articular cartilage mostly caused by proliferative synovial fibroblasts of the pannus (*23*). It is likely that, besides cathepsin L and B (*24*, *25*), cathepsin K also contributes to the massive cartilage destruction in this debilitating disease.

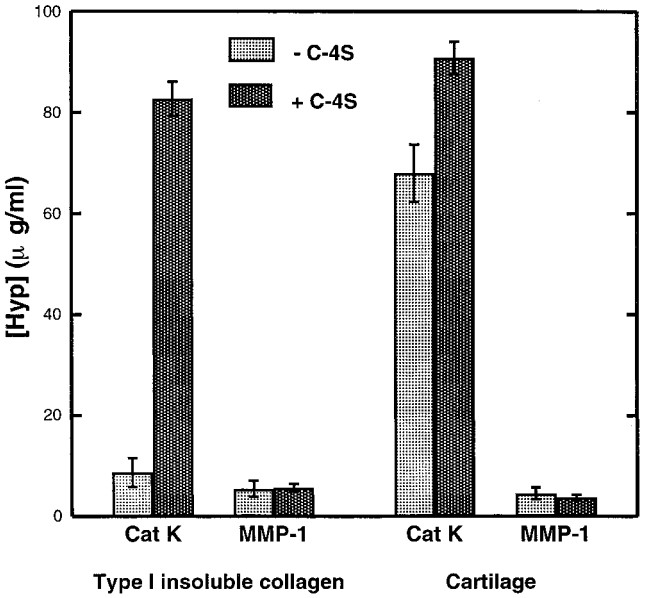

FIGURE 6: Comparison of hydrolysis rates of insoluble type I bovine collagen and bovine cartilage by cathepsin K and MMP-1 in the absence or the presence of C-4S. Insoluble type I bovine collagen (3.5 mg/mL) and bovine cartilage (45 mg/mL) were digested in the absence or the presence of 0.15% C-4S at 28 °C for 24 h with 800 nM cathepsins K at pH 5.5, or with 400 nM MMP-1 at pH 7.2. The reaction samples were pelleted and the hydroxyproline concentration of supernatant, representing the released collagen fragments, was determined. The data represent the mean ± SD of four experiments per digestion condition.

Table 3: p*K* Values of pH Activity Profiles for Cathepsin K in the Presence and Absence of Chondroitin 4-Sulfate

	pK_1	pK_2	pH optimum	pK_2−pK_1
0% GAGs	3.68 ± 0.1	7.87 ± 0.09	5.78	4.19
0.15% C-4S	3.48 ± 0.06	7.97 ± 0.05	5.73	4.49

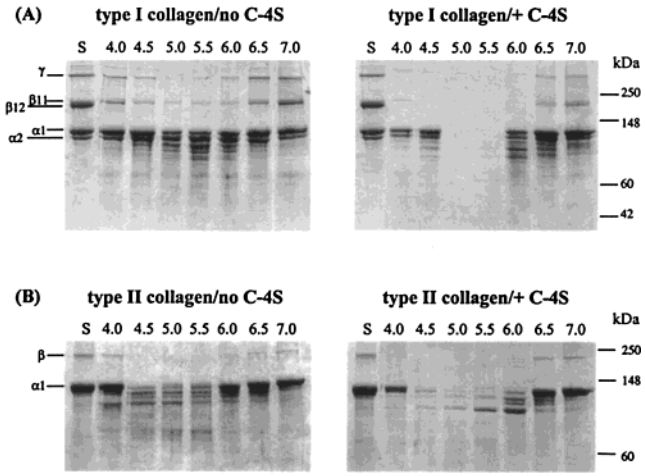

FIGURE 7: SDS−PAGE analysis of pH collagenolytic activity profile for recombinant human cathepsin K at the absence and the presence of C-4S. Soluble type I or II bovine collagen was incubated with 800 nM cathepsin K at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 (shown in the top margin) in the absence (for 12 h.) or the presence (for 6 h) of 0.15% C-4S. Untreated soluble collagen was used as standard (S). Molecular mass standards are indicated in the right lane.

The effect of GAGs on the activity and stability of cathepsin K is most likely due to specific interactions between the protease and the GAGs and not between the

GAGs and the substrate. GAG−substrate interactions would imply substrate-specific alteration of the accessibility to protease hydrolysis. Arguments against the GAG−substrate interactions are that the degradation of native triple helical collagen and denatured collagen (gelatin) are both enhanced in the presence of GAGs and that no effect was observed on the activities of cathepsin L and MMP-1. Since the presence of GAGs in the reaction mixture have only minor or no effects on the catalytic constants of cathepsin K hydrolysis rates and the glycosaminoglycans clearly increase the stability of the cathepsin K protein, interactions between the protease and the GAG must have a conformation-stabilizing character. Direct binding of GAGs to proteases has been previously described. Kainulainen et al. (*26*) reported binding constants of a heparan−sulfate proteoglycan in the high nanomolar range to the serine protease cathepsin G. Furthermore, the inhibition of leukocyte elastase-sulfated GAGs has been documented (*27*). Future studies will investigate the specificity of the binding interactions for different sulfated GAGs to individual cathepsins using binding assays and modeling experiments correlating the surface area potential of the proteases to the negatively charged GAGs.

REFERENCES

1. Carney, S. L., and Muir, H. (1988) *Physiol. Rev. 68*, 858−910.
2. Shinmei, M., Miyauchi, S., Machida, A., and Miyazaki, K. (1992) *Arthritis Rheum. 35*, 1304−1308.
3. Brömme, D., and Okamoto, K. (1995) *Biol. Chem. Hoppe-Seyler 376*, 379−384.
4. Tezuka, K., Nemoto, K., Tezuka, Y., Sato, T., Ikeda, Y., Kobori, M., Kawashima, H., Eguchi, H., Hakeda, Y., and Kumegawa, M. (1994) *J. Biol. Chem. 269*, 15006−15009.
5. Drake, F. H., Dodds, R. A., James, I. E., Connor, J. R., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M. (1996) *J. Biol. Chem. 271*, 12511−12516.
6. Hummel, K. M., Petrow, P. K., Franz, J. K., Muller-Ladner, U., Aicher, W. K., Gay, R. E., Brömme, D., and Gay, S. (1998) *J. Rheumatol. 25*, 1887−1894.
7. Kafienah, W., Bromme, D., Buttle, D. J., Croucher, L. J., and Hollander, A. P. (1998) *Biochem. J. 331*, 727−732.
8. Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F. H., McQueney, M. S., Foged, N. T., Delmas, P. D., and Delaisse, J. M. (1998) *J. Biol. Chem. 273*, 32347−32352.
9. Gelb, B. D., Shi, G. P., Chapman, H. A., and Desnick, R. J. (1996) *Science 273*, 1236−1238.
10. Hou, W.-S., Brömme, D., Zhao, Y., Mehler, E., Dushey, C., Weinstein, H., Miranda, C. S., Fraga, C., Greig, F., Carey, J., Rimoin, D. L., Desnick, R. J., and Gelb, B. D. (1999) *J. Clin. Invest. 103*, 731−738.
11. Brömme, D., Steinert, A., Friebe, S., Fittkau, S., Wiederanders, B., and Kirschke, H. (1989) *Biochem. J. 264*, 475−481.
12. Linnevers, C. J., McGrath, M. E., Armstrong, R., Mistry, F. R., Barnes, M., Klaus, J. L., Palmer, J. T., Katz, B. A., and Brömme, D. (1997) *Protein Sci. 6*, 919−921.
13. Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., and Hanada, K. (1982) *Biochem. J. 201*, 189−198.
14. Leatherbarrow, R. J. (1987) Elsevier Biosoft, Cambridge, U.K.
15. Heinegard, D. (1972) *Biochim. Biophys. Acta 285*, 181−192.
16. Khouri, H. E., Vernet, T., Menard, R., Parlati, F., Laflamme, P., Tessier, D. C., Gour- Salin, B., Thomas, D. Y., and Storer, A. C. (1991) *Biochemistry 30*, 8929−8936.
17. Firschein, H. E., and Shill, J. P. (1966) *Anal. Biochem. 14*, 296−304.
18. Bromley, M., Bertfield, H., Evanson, J. M., and Woolley, D. E. (1985) *Ann. Rheum. Dis. 44*, 676−681.
19. Menard, R., Khouri, H. E., Plouffe, C., Laflamme, P., Dupras, R., Vernet, T., Tessier, D. C., Thomas, D. Y., and Storer, A. C. (1991) *Biochemistry 30*, 5531−5538.
20. Everts, V., Aronson, D. C., and Beertsen, W. (1985) *Calcif. Tissue Int. 37*, 25−31.
21. Harab, R. C., and Mourao, P. A. (1989) *Biochim. Biophys. Acta 992*, 237−240.
22. Deutsch, A. J., Midura, R. J., and Plaas, A. H. (1995) *J. Orthop. Res. 13*, 230−239.
23. Firestein, G. S. (1996) *Arthritis Rheum. 39*, 1781−1790.
24. Trabandt, A., Aicher, W. K., Gay, R. E., Sukhatme, V. P., Nilson-Hamilton, M., Hamilton, R. T., McGhee, J. R., Fassbender, H. G., and Gay, S. (1990) *Matrix 10*, 349−361.
25. Trabandt, A., Gay, R. E., Fassbender, H.-G., and Gay, S. (1991) *Arthritis Rheum. 34*, 1444−1451.
26. Kainulainen, V., Wang, H., Schick, C., and Bernfield, M. (1998) *J. Biol. Chem. 273*, 11563−11569.
27. Paroli, E. (1993) *Int. J. Clin. Pharmacol. Res. 13*, S1−S9.

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