Age-related Changes in Aggrecan Glycosylation Affect Cleavage by Aggrecanase*

Michael A. Pratta, Micky D. Tortorella, and Elizabeth C. Arner

From Inflammatory Diseases Research, The DuPont Pharmaceutical Company, Experimental Station Wilmington, Delaware 19880-0400

Aggrecan degradation involves proteolytic cleavage of the core protein within the interglobular domain. Because aggrecan is highly glycosylated with chondroitin sulfate (CS) and keratan sulfate (KS), we investigated whether glycosylation affects digestion by aggrecanase at the Glu373–Ala374 bond. Treatment of bovine aggrecan monomers to remove CS and KS resulted in loss of cleavage at this site, suggesting that glycosaminoglycans (GAGs) play a role in cleavage at the Glu373–Ala374 bond. In contrast, MMP-3 cleavage at the Ser341–Phe342 bond was not affected by glycosidase treatment of aggrecan. Removal of KS, but not CS, prevented cleavage at the Glu373–Ala374 bond. Thus, KS residues may be important for recognition of this cleavage site by aggrecanase. KS glycosylation has been observed at sites adjacent to the Glu373–Ala374 bond in steer aggrecan, but not in calf aggrecan (Barry, F. P., Rosenberg, L. C., Gaw, J. U., Gaw, J. U., Koob, T. J., and Neame, P. J. (1995) J. Biol. Chem. 270, 20516–20524). Interestingly, although we found that aggrecanase degraded both calf and steer cartilage aggrecan, the proportion of fragments generated by cleavage at the Glu373–Ala374 bond was higher in steer than in calf, consistent with our observations using aggrecan treated to remove KS. We conclude that the GAG content of aggrecan influences the specificity of aggrecanase for cleavage at the Glu373–Ala374 bond and suggest that age may be a factor in aggrecanase degradation of cartilage.

The articular cartilage matrix consists primarily of collagen, which provides strength and support, and proteoglycan, which contributes qualities of compressibility and elasticity. The major type of proteoglycan present in cartilage is aggrecan, which is composed of a protein core that contains a high level of sulfated glycosaminoglycans (GAGs), both CS and KS. The sulfate groups of these sugar molecules impart a net negative charge to aggrecan thus providing the attractive forces that incorporate water into the matrix and endow the tissue with its shock-absorbing quality.

The aggrecan core protein contains three globular domains; G1, through which the molecule binds to hyaluronic acid, G2, and G3 (1, 2). In diseased tissue, the matrix is lost, and this loss is associated with degradation of the aggrecan monomers (3). The interglobular domain (IGD) of the aggrecan core protein, which is located between G1 and G2, contains proteolytic cleavage sites that are believed to be critical to the overall loss of aggrecan function. Two major sites of digestion have been identified within the IGD between residues Asn341 and Phe342 and between Glu373 and Ala374. The first site has been shown to be cleaved by a variety of matrix metalloproteinases (MMPs) (4–8), whereas cleavage at the second site is catalyzed by aggrecanase. The contribution of aggrecanase to aggrecan cleavage has been investigated based on the generation of products that terminate with Glu373 or begin with Ala374. Several reports have shown that the majority of the aggrecan fragments found both in vitro in response to stimulated cartilage degradation (9–13) and in vivo in arthritic synovial fluids (14, 15) are generated by cleavage at the aggrecanase site.

Recent in vitro studies from our laboratory (16) show that there is a strong correlation between specific cleavage at the Glu373–Ala374 bond and the release of aggrecan catabolites in response to cytokine stimulation. In addition, the ability of inhibitors to block the release of aggrecan catabolites correlates with their ability to block specific cleavage at the aggrecanase site. Taken together these data suggest that aggrecanase plays a key role in aggrecan degradation. Thus, identifying factors that influence the ability of aggrecanase to cleave cartilage aggrecan is important for understanding the regulation of aggrecan catabolism by this enzyme in both normal matrix turnover and in arthritic disease.

Because the aggrecan core protein is heavily glycosylated, it is possible that the glycosaminoglycans on the aggrecan molecule may affect the ability of either MMPs or aggrecanase to cleave the core protein. This notion is especially intriguing based on the finding by Barry et al. (17) that glycosylation of aggrecan in articular cartilage is altered with age and thus could potentially play a role in regulating the susceptibility of aggrecan to degradation during aging. These authors reported that aggrecan isolated from steer (age of 1.5–2 years) articular cartilage is post-translationally modified with a keratan sulfate within the sequence 368NITEGEAR375 whereas aggrecan from calf (age of 1 week) articular cartilage lacks glycosylation in this region. Because this sequence contains the aggrecanase cleavage site, the presence of glycosylation may play a role in regulating aggrecanase-mediated degradation. Studies by Bayliss and co-workers (18) detected aggrecan G1 fragments with the C terminus (ITEGE), indicating that they had been cleaved.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Inflammatory Diseases Research, The DuPont Pharmaceutical Company, Experimental Station Wilmington, Delaware 19880-0400.
at the aggrecanase site in normal adult human articular cartilage but not in immature articular cartilage, and the amount of these aggrecanase-generated fragments increased with age. These data are consistent with the possibility that aggrecanase can cleave adult aggrecan more readily than the immature aggrecan, which lacks glycosylation in the region surrounding the aggrecanase cleavage site. Therefore, we investigated the influence of glycosylation on aggrecan digestion by aggrecanase.

We have recently generated soluble aggrecanase activity in media from IL-1 stimulated bovine nasal cartilage and developed an enzymatic assay using purified bovine aggrecan monomers as substrate (19). Products were monitored by Western analysis using the monoclonal antibody, BC-3, which recognizes the N terminus of the aggrecanase-generated products with the sequence $\text{ARGSVIL}^{374}$. In this report, we demonstrate that deglycosylation of the aggrecan substrate results in total inhibition of BC-3-reactive product generation by aggrecanase, suggesting that GAGs play a role in aggrecanase-mediated cleavage at the Glu$^{373}$–Ala$^{374}$ bond. Further, we show that aggrecanase differs in the ability to cleave at the Glu$^{373}$–Ala$^{374}$ bond in steer versus calf aggrecan, suggesting that the changes in glycosylation with age may influence the ability of aggrecan to be cleaved at the Glu$^{373}$–Ala$^{374}$ site by aggrecanase.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fresh bovine nasal and articular cartilage were obtained from Co-vance Inc., (Denver, PA). Guanidine hydrochloride was from Pierce Chemical Co. (Rockford, IL). Cesium chloride and $\beta$-mercaptoethanol were from Sigma (St. Louis, MO). Dulbecco’s modified Eagle’s medium, Antibiotic-Antimycotic and neomycin sulfate were from Life Technologies, Inc. (Grand Island, NY). The IL-1 used was a soluble fully active recombinant human IL-1β produced as described previously (20). The specific activity was $1 \times 10^7$ units/mg, with 1 unit being defined as the amount of enzyme that converts 1 pmole of substrate per minute at 37°C. Recombinant human IL-1β (100 units/ml) was from the manufacturer (1 tablet/50 ml). The mixture was shown to have no effect on the IgG products generated from calf (≤3 months) or steer (>9 months) nasal or articular cartilage by extraction in 4 M guanidine hydrochloride and separation by cesium chloride density gradient centrifugation (25). Gradient fractions with a density greater than 1.5 g/ml containing aggrecan monomers were dialyzed into water and lyophilized. Prior to enzymatic assay, aggrecan was resuspended in water at a concentration of 2 mg/ml (w/v). Calf and steer aggrecan preparations were found to be similar with respect to GAG concentration (steer: $1630 \pm 71$ μg/ml; calf: $1599 \pm 51$ μg/ml) as well as to staining in an anti-G1 Western analysis (described below).

**Preparation of Deglycosylated Aggrecan Substrate**—Deglycosylated aggrecan substrate was prepared by incubating bovine aggrecan monomers with chondroitinase ABC (0.1 units/10 μg), keratanase (0.1 units/10 μg), and keratanase II (100 units/10 μg) for 4 h at 37°C in 100 mM Tris/HCl with 100 mM sodium acetate, pH 6.5. Deglycosylation was confirmed by monitoring GAG concentration using the dimethyl-methylene blue (DMMB) assay (27). Aggrecan incubated with heat-inactivated glycosidases served as a control. There was no difference in data obtained using fully glycosylated substrate, and this same substrate incubated with boiled deglycosylase enzymes (data not shown). Isolation of aggrecan core from digestion products by acetone precipitation did not alter results (data not shown), suggesting that the GAG fragments generated do not affect digestion by aggrecanase.

**Aggrecanase Digestion**—A crude preparation of aggrecanase was generated in conditioned media from bovine nasal cartilage stimulated over a 14-day culture period with human recombinant IL-1β (500 ng/ml) (19, 26). Because this preparation of aggrecanase also contained MMPs, we were concerned that it potentially interfered with evaluation of aggrecanase activity. Therefore, we incubated calf aggrecan, keratanase II (1000 units/10 μg), and MMP-3, aggrecan and aggrecan fragments in the reaction mixture to be cleaved at the Glu$^{373}$–Ala$^{374}$ site by aggrecanase.

**Isolation of aggrecan core from digestion products**—Aggrecan fragments released from the freeze/thawed tissue were determined colorimetrically by glycosaminoglycan assay (27). Products of the enzymatic reaction were detected by Western analysis (19, 26). A pre-quenched sample in which aggrecanase was treated with EDTA (20 μM) prior to incubation with substrate represents background levels of the BC-3-reactive fragments present in the aggrecanase preparation. Aggrecan (either native or deglycosylated) seeded in buffer incubated for up to 4 h at 37°C did not significantly affect staining of the pre-quenched sample (data not shown). In previous studies, buffer from freezethawed steer cartilage incubated for 0–48 h was analyzed by BC-3 and AF-28 Western analysis, as well as for GAG release (31). Although low levels of GAG release occurred over this time period, it was likely because of non-enzymatic diffusion of aggrecan from the cut surfaces of the cartilage as no BC-3 or AF-28 reactive fragments were detected.

**MMP-3 Digestion**—Recombinant C-terminally truncated pro-MMP-3 was activated for 24 h at 37°C in the presence of 1 mM 4-amino-phenylmercuric acetate (APMA) and dialyzed overnight at 4 °C into 50 mM Tris/HCl with 100 mM sodium acetate, pH 7.0. Aggrecan was digested with tMMP-3 as described above using 50 nm of the isolated half-mature aggrecan.

**Western Blot Analysis**—Following digestion by aggrecanase or tMMP-3, agarose and agarose agar fragments in the reaction mixture were deglycosylated with chondroitinase ABC, keratanase, and keratanase II as described above, with the addition of a protease inhibitor mixture (COMPLETE*). Based on conditions recommended by the manufacturer (1 tablet/50 ml). The mixture was shown to have no effect on the generation of the aggrecanase digestion enzymes (data not shown). The samples were then precipitated with ice-cold acetone for 15 min, centrifuged (14000 × g) for 5 min at 4 °C, and the supernatant was removed by aspiration. The pellet was dried under nitrogen and solubilized in SDS-polyacrylamide gel electrophoresis loading buffer containing 2.5% β-mercaptoethanol. Proteins were separated by SDS-polyacrylamide gel electrophoresis using a 4–12% gradient gel and then transferred to polyvinylidene difluoride overnight at 30 V in Trisglycine buffer.
Glycosaminoglycan Assay—GAG levels were determined based on the amount of polyanionic material reacting with DMBB using shark chondroitin sulfate as standard (27).

RESULTS
Effect of Aggrecan Glycosylation on Digestion by Aggrecanase—Aggrecanase digestion of native steer aggrecan resulted in the generation of several BC-3-reactive products (Fig. 1A, lane 4), whereas deglycosylation of the aggrecan substrate resulted in a complete loss of BC-3 reactive fragment generation (Fig. 1A, lane 2). However, evaluation of these same samples by CSPG Western analysis (Fig. 1B) indicated depletion of intact aggrecan based on the loss of staining of high molecular mass proteins (>250 kDa; Fig. 1B, lane 2). These data suggest that the deglycosylated aggrecan substrate is cleaved by aggrecanase at an alternative site. This conclusion is supported by the observation that the products formed are different when compared with the fully glycosylated substrate (Fig. 1B, lane 4). For example, an aggrecan fragment produced upon cleavage of glycosylated aggrecan (lane 4, arrowhead a) was not detected when substrate was deglycosylated (lane 2). In contrast, a fragment produced by cleavage of deglycosylated aggrecan (lane 2, arrowhead b) was absent when glycosylated aggrecan was used. No AF-28 reactive fragments were detected with either native or deglycosylated substrate (data not shown), indicating that deglycosylation does not facilitate aggrecanase cleavage at the Ser341–Phe342 MMP site.

Next, the effect of glycosylation on cleavage of bovine aggrecan at the MMP cleavage site (Ser341–Phe342) was assessed using TMMP-3. No difference in the generation of AF-28-reactive products was detected using deglycosylated as compared with fully glycosylated aggrecan (Fig. 2). Consistent with these findings, CSPG Western analysis showed no difference with deglycosylation of the aggrecan substrate (data not shown). Finally, MMP-3 did not cleave at the aggrecan site (Glu373–Ala374) using fully glycosylated or deglycosylated aggrecan as substrate (data not shown).

To study the individual roles of keratan sulfate and chondroitin sulfate in cleavage at the Glu373–Ala374 bond, we treated aggrecan from articular cartilage individually either with chondroitinase ABC or with keratanase and keratanase II. The use of keratanase-treated aggrecan as substrate resulted in complete loss of cleavage at the Glu373–Ala374 bond similar to fully deglycosylated aggrecan, suggesting a role for keratan sulfate in the recognition of the Glu373–Ala374 cleavage site by aggrecanase (Fig. 3A). When chondroitinase ABC-treated aggrecan was used as substrate, products were generated by cleavage at the Glu373–Ala374 bond (Fig. 3B), although several higher molecular mass products (>250 kDa; indicated by arrowheads at the 4-h time point) were also generated. Similar results were obtained using aggrecan isolated from nasal cartilage (data not shown).

Effect of Age on Aggrecanase Digestion of Articular Cartilage—Barry et al. (17) demonstrated that aggrecan isolated from steer articular cartilage is post-translationally modified with a keratan sulfate within the sequence 660NITEGEAR375 whereas calf aggrecan lacks glycosylation in this area. Because this region contains the aggrecanase cleavage site, we evaluated digestion of calf and steer cartilage by aggrecanase. Freeze-thawed articular cartilage from the metacarpophalangeal joint of calf (<3 months) or steer (>9 months) was used as substrate for aggrecanase. Overall aggrecan cleavage as measured by total GAG release over time was similar from calf and steer articular cartilage (Fig. 4A). Because the aggrecanase preparation also contains MMPs (19), which could contribute to the GAG release shown in Fig. 4A, the effect of aggrecanase digestion of calf and steer cartilage was evaluated in the presence of the MMP inhibitor, XS309 (100 nM), and results are shown in Fig. 4B. Aggrecanase digestion of steer cartilage was
not affected by the inclusion of XS309, consistent with the hypothesis that the enzyme responsible for the majority of GAG release from steer cartilage is aggrecanase. In contrast, inclusion of XS309 in the aggrecanase digestion of calf cartilage resulted in a significant decrease in GAG release suggesting that MMPs are playing a prominent role in the GAG release from calf cartilage. Evaluation of aggrecan fragments from both calf and steer cartilage in an AF-28 Western blot showed that a larger portion of the GAG release was derived from MMP digestion in calf than in steer and that the generation of the AF-28-reactive fragments was blocked by XS309 in both tissues (Fig. 4C).

Evaluation of digests by BC-3 Western analysis indicated that there was a striking difference in the amount of BC-3-reactive products generated (Fig. 4D). Although the amount of GAG loaded in each lane was similar (5 µg/lane), the amount of BC-3 reactive products released from steer cartilage was several-fold greater than that released from calf cartilage. Thus, when BC-3-reactive products were quantitated and expressed per µg of GAG, levels at the 16-h time point were found to be at least 4-fold greater for steer than for calf cartilage. Taken together, these data suggest that both calf and steer aggrecan are digested by aggrecanase, but steer aggrecan is cleaved more efficiently at the Glu373-Ala374 bond.

Because the studies in Fig. 4 were performed using intact cartilage, diffusion of aggrecanase into the cartilage, as well as the release of aggrecan fragments from the cartilage, may influence the results. To eliminate the issue of diffusion and to further evaluate aggrecanase-mediated cleavage of calf and steer aggrecan, we isolated aggrecan monomers from calf and steer articular cartilage and digested them with aggrecanase. Digests were then evaluated with an antibody to the NITEGE C terminus as well as with the BC-3 antibody to the ARGSV N terminus (Fig. 5). 3- to 5-fold higher levels of BC-3-reactive fragments were produced by aggrecanase digestion of steer articular aggrecan relative to calf (Fig. 5A), similar to that found with freeze/thawed calf cartilage (Fig. 4D). Consistent with these results, there were increased levels of G1-NITEGE fragments produced in response to aggrecanase digestion of steer versus calf aggrecan as demonstrated by a doublet between 64–70 kDa (13) in Fig. 5B. Similar results were obtained when aggrecan digested with aggrecanase-1 (ADAMTS-4) was analyzed by anti-G1 Western (data not shown), confirming the identity of the doublet in Fig. 5B. Analysis by scanning densitometry of anti-G1 Western blots containing undigested calf and steer aggrecan (Fig. 5C) indicated that the intensity of overall staining of the steer was 1.5-fold higher than that of the calf, suggesting that the differences in BC-3-staining in Fig. 5A cannot be attributed solely to the differences in core protein levels of the calf and steer aggrecan. Although the distribution of G1-containing fragments differed in undigested calf and steer aggrecan, these differences were probably because of age-related C-terminal truncations. Thus, these data support the hypothesis that aggrecan glycosylation plays a role in cleavage by aggrecanase at the Glu373-Ala374 bond.

**DISCUSSION**

Our data suggest a potential role for aggrecan glycosylation as a regulatory mechanism for aggrecanase-mediated cleavage. In this report, we have demonstrated the following. 1) Aggrecanase generation of BC-3 reactive aggrecan fragments formed by specific cleavage at the Glu373-Ala374 bond was eliminated with up to a 4-h digestion by prior deglycosylation of the aggrecan substrate. 2) Aggrecanase digestion of aggrecan in freeze/thawed calf cartilage, which lacks a keratan sulfate residue near the aggrecanase cleavage site that is present in steer aggrecan (17), resulted in the generation of dramatically less BC-3 reactive fragments, and 3) digestion of isolated calf aggrecan by aggrecanase resulted in the generation of lower levels of both BC-3-reactive fragments with the ARGSV N terminus and G1 fragments with the NITEGE C terminus relative to the levels generated using isolated steer aggrecan.

Although deglycosylation of the aggrecan substrate eliminated the generation of BC-3 reactive fragments representing cleavage at the Glu373-Ala374 bond within the IGD, the deglycosylated substrate was cleaved by aggrecanase as evidenced by depletion of intact aggrecan as monitored by CSPG Western blot analysis. However, the fragments produced were of different sizes than those generated using the fully glycosylated substrate. Thus, aggrecanase appears to cleave the aggrecan core protein at different sites when the substrate lacks GAG side chains. Several additional aggrecanase-susceptible sites have been identified within the C-terminal domain of aggrecan (29). Cleavage at these sites may be responsible for the alternative products produced with the deglycosylated aggrecan substrate. Conversely, other sites within the IGD may become susceptible to cleavage by aggrecanase following deglycosylation. Although the sites of cleavage in the deglycosylated substrate were not identified in the current studies, examination of the digests by Western blot analysis using the AF-28 antibody that recognizes the FFGVG N terminus generated by cleavage at the Ser341-Phe342 MMP site indicated that deglycosylation did not result in cleavage by aggrecanase at this site (data not shown).

It has been reported that the GAG digestion enzyme preparations may contain low levels of contaminating proteases, and the use of a protease inhibitor mixture is recommended to eliminate any effect by contaminants on sample integrity (30). The protease inhibitor mixture included during deglycosylation of samples following enzymatic assay could not be used in the preparation of the deglycosylated substrate because the presence of the mixture would interfere with aggrecanase and tMMP-3 enzyme activities. However, two pieces of data suggest that the involvement of contaminating proteases in the current studies, if any, is minimal. First, in the CSPG Western analysis shown in Fig. 1B, there was no apparent difference in staining between deglycosylated and native aggrecan substrate (lanes 1 versus 3), indicating that treatment during deglycosylation does not appreciably alter the core protein of aggrecan. Second, if the aggrecan core were degraded, one would expect the AF-28
Western analysis of MMP-3-digested deglycosylated aggrecan and native aggrecan (Fig. 2) to be different. The fact that there was no apparent difference in fragment pattern between deglycosylated and native substrate support the conclusion that the aggrecan core is not effected by deglycosylation treatment, and therefore, the differences noted are because of differences in glycosylation of the substrate.

Our data using deglycosylated aggrecan as a substrate suggest that the ability of aggrecanase to cleave at the Glu\textsuperscript{373}–Ala\textsuperscript{374} site within the IGD of aggrecan is dependent on the presence of GAGs that are associated with the aggrecan core protein. However, aggrecan from rat chondrosarcoma that lacks KS is cleaved at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond, and this cleavage is presumably because of aggrecanase (13). In addition,
cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond by aggrecanase was detected using recombinant substrates lacking KS (28, 34). These data suggest that the presence of KS is not an absolute requirement for cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond, but rather that the presence of KS may increase the efficiency of cleavage at this site. There may also be differences in the cleavage mechanism of a relatively small peptide substrate and native aggrecan. Because we have recently reported cloning and expression of two proteases that possess aggrecanase activity (32, 33), these mechanisms can now be investigated.

Studies to dissect out the individual roles of the CS and KS chains in regulating aggrecanase cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond indicated that removal of only the KS chains from the aggrecan substrate by keratanase digestion resulted in a complete loss of cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond as evidenced by the lack of BC-3 fragment generation. These data implicate KS as playing an important role in cleavage at this site by aggrecanase. This hypothesis is supported by our data showing that aggrecanase digestion of calf aggrecan, which lacks a KS residue that is present in steer aggrecan near the aggrecanase cleavage site (17), resulted in lower levels of BC-3-reactive fragments than produced when steer aggrecan was used as the substrate. When only the CS chains were removed by chondroitinase ABC digestion, cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond was not eliminated. This is consistent with the reported lack of CS chains within the IGD. Interestingly, with chondroitinase-treated aggrecan substrate, some products formed by cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond were larger than those generated using the fully glycosylated substrate. We have recently demonstrated that aggrecanase-1 (35) and aggrecanase-2\textsuperscript{2} cleave aggrecan at four additional sites within the C terminus of the molecule. Decreased cleavage at these C-terminal sites would be expected to result in the generation of larger fragments upon cleavage at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond. Because these sites are in regions surrounded by multiple CS chains, it is possible that loss of CS may be affecting aggrecanase cleavage within the C terminus of the aggrecan molecule.

In contrast to the dramatic effect of glycosylation on cleavage by aggrecanase at the Glu\textsuperscript{373}–Ala\textsuperscript{374} bond, MMP-3 digestion of bovine aggrecan at the Ser\textsuperscript{341}–Phe\textsuperscript{342} appeared unaffected by glycosylation state. Although it could be hypothesized that the presence of KS on the fully glycosylated steer aggrecan protects this substrate from cleavage by MMPs present in the native aggrecanase preparation, and in turn, results in increased substrate for aggrecanase-mediated cleavage, several pieces of data indicate that this is not the case: 1) the finding from our laboratory that this data can be reproduced using recombinant ADAMTS-4 (23), material that would be devoid of MMP activity; 2) the MMP inhibitor, X5390, that was included in these studies to eliminate the contribution of any contaminating MMPs to cleavage; and 3) no change was observed in the ladder of AF-28-reactive products, and no differences were detected by CSPG Western analysis of the products generated upon digestion of glycosylated vs deglycosylated aggrecan by MMP-3. This suggests that glycosylation did not affect digestion at the Ser\textsuperscript{341}–Phe\textsuperscript{342} bond or at other MMP-3 cleavage sites on the C-terminal side of the Ser\textsuperscript{341}–Phe\textsuperscript{342} bond.

A potential hypothesis for these observations may be related to differences in the presence of glycosylation surrounding the respective cleavage sites. Whereas the aggrecan core protein has been shown to be post-translationally modified with a keratan sulfate residue near the Glu\textsuperscript{373}–Ala\textsuperscript{374} aggrecanase cleavage site, there is no evidence of similar glycosylation on residues surrounding the MMP cleavage site (17). Therefore, deglycosylation would be less likely to affect MMP-mediated aggrecan digestion. Another potential explanation is that substrate recognition by aggrecanase may be affected by GAGs whereas recognition by MMP-3 may not, possibly because of structural differences between the two proteases. Consistent with this hypothesis, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5), the proteases that we recently cloned and expressed, contain C-terminal thrombospondin-1-like motifs (32, 33) that appear to be important for recognition of the aggrecan substrate through interaction with GAG chains (23).

Evaluation of GAG release (Fig. 4) provided additional evidence that support the hypothesis that steer aggrecan in the presence or absence of an MMP inhibitor is more readily digested by aggrecanase than calf aggrecan. Because calf aggrecan is less susceptible to aggrecanase digestion, a larger amount would be available as a substrate for MMPs present in the aggrecanase preparation. Consistent with this hypothesis, MMP-mediated cleavage appears to be contributing signifi-

\textsuperscript{2} M. D. Tortorella, R-Q. Liu, M. Pratta, O. H. Ross, I. Abbaszade, T. Burn, H. Nagase, and E. C. Arner, unpublished data.
cantly to the GAG release from cartilage in the absence of the MMP inhibitor, XS309 (Fig. 4A). AF-28 Western analysis indicated that a larger portion of the GAG release was derived from MMP digestion in the calf than in the steer under these conditions (Fig. 4C). In addition, MMP-generated GAG release in the steer is inhibited by XS309 and results in a reduction in overall GAG release from the tissue (Fig. 4D). However, GAG release from steer cartilage is not greatly affected by the presence of XS309, suggesting that GAG release from steer cartilage is largely because of aggrecanase-mediated cleavage. This observation is consistent with the observation that digestion of steer cartilage by aggrecanase resulted in higher levels of BC-3-reactive fragments than in the calf (Fig. 4D). Additional evidence in support of this data is that recombiant aggrecanase1 (ADAMTS-4), like native aggrecanase, shows a preference for steer versus calf aggrecan (data not shown), as well as similar sensitivity to substrate glycosylation (23).

Whereas both aggrecanase and MMPs have been implicated in proteoglycan degradation, the differences in their sensitivity to aggrecan glycosylation may suggest different roles for these enzymes in aggrecan turnover. The finding by Barry et al. (17) that glycosylation differences occur near the aggrecanase cleavage site in the aggrecan IGD relative to age opens the possibility that glycosylation may be playing a role in regulating changes in aggrecanase-mediated cleavage. Consistent with this theory is the finding that higher levels of BC-3-reactive fragments are generated by aggrecanase digestion from steer than from calf articular cartilage. It is interesting to speculate that the additional KS residue near the aggrecanase cleavage site, which is associated with advanced age, plays a permissive role for digestion at this site by aggrecanase in the mature animal.

Our data indicate that glycosylation of aggrecan represents a potential regulatory mechanism for aggrecanase-mediated proteoglycan degradation. Because changes in aggrecan glycosylation with age have been demonstrated in regions of the core protein specific for cleavage by aggrecanase, this may serve as a means of controlling the relative rate of cleavage within the IGD by this protease with maturation and age.

Acknowledgments —We are grateful to Dr. Ada Cole and Barbara L. Schumacher (Rush Presbyterian-St. Luke’s Medical Center, Chicago, IL) for NITTEGE Western blot analysis. We thank Dr. Carl Decicco and the DuPont Medicinal Chemistry Department for the synthesis of XS309, Dr. Robert Copeland for MMP inhibitors, L. Davis, G., and Maniglia, C. A. (1992) Biochem. J. 284, 589–593.

Illic, M. Z., Handley, C. J., Robinson, H. C., and Mok, M. T. (1992) Arch. Biochem. Biophys. 294, 174–178.

Lark, M. W., Gyordy, J. T., Weidner, J. R., Ayala, J., Kimura, J. H., Williams, H. R., Mumford, R. A., Flannery, C. R., Carlson, S. S., Iwata, M., and Sandy, J. D. (1995) J. Biol. Chem. 270, 2550–2556.

Sandy, J. D., Flannery, C. R., Neame, P. J., and Lohmander, L. S. (1992) J. Clin. Invest. 89, 1512–1516.

Lohmander, L. S., Neame, P. J., and Sandy, J. D. (1993) Arthritis Rheum. 36, 1214–1222.

Arner, E. C., Hughes, C. E., Decicco, C. P., Carter, B., and Tortorella, M. D. (1998) Osteoarthrits Cartilage 6, 214–228.

Barry, P. F., Rosenberg, L. C., Gao, J. U., Gao, J. U., Koo, T. B., and Neame, P. J. (1995) J. Biol. Chem. 270, 20516–20524.

Hutton, S. E., Haward, J., Maciewicz, R., and Bayliss, M. T. (1996) Trans. Orthop. Res. Soc. 21, 150.

Arner, E. C., Pratta, M. A., Trzaskos, J. M., Decicco, C. P., and Tortorella, M. D. (1998) J. Biol. Chem. 273, 6594–6601.

Huang, J. J., Newton, R. C., Pezzella, K., Covic, M., Tamblin, T., Rutlege, S., Gray, J., Kelly, M., and Liu, Y. (1987) Mol. Biol. Med. 4, 169–181.

Hughes, C. E., Carter, B., Fosang, A. J., Roughley, P. J., and Mert, J. S. (1995) Biochem. J. 303, 799–804.

Sandy, J. D., Kast, K., Gardiner, P., Jackson, D. C., and Brown, L. (1995) Biochem. J. 310, 337–343.

Tortorella, M. D., Pratta, M. A., Liu, R. Q., Abbazsade, I., Ross, O. H., Burt, T., and Arner E. C. (2000) J. Biol. Chem. 275, 25791–25797.

Marcy, A. L., Elberger, L. L., Harrison, R., Chan, H. K., Hutchinson, N. L., Hagman, W. K., Cameron, P. M., Boulton, D. A., and Hermes, J. D. (1991) Biochemistry 30, 6476–6483.

Hasel, C. V., and Kimura, H. (1982) Methods Enzymol. 82, 769–800.

Xue, C.-R., Cheryne, R. J., DeCicco, C. P., DeGrado, W. F., He, X., Hodge, C. N., Jacobsen, I. C., Magolda, R. L., and Arner, E. C. (May 22, 1997) WO 1997/021742.

Farnblade, R. W., Sayers, C. A., and Barrett, A. J. (1992) Connect. Tissue Res. 9, 247–248.

Hughes, C. E., Buttner, F. H., Eidenmuller, B., Carteson, B., and Bartnik, E. N. (1997) J. Biol. Chem. 272, 20269–20274.

Sandy, J. D., Plaa, A. H. K., and Koo, T. J. (1995) Acta Orthop. Scand. Suppl. 266, 26–32.

Oike, Y., Kimata, K., Shinomura, T., and Suzuki, S. (1980) Biochem. J. 191, 203–207.

Arner, E. C., Hughes, C. E., Decicco, C. P., Xue, C. E., Newton, R. C., Trzaskos, J. M., Magolda, R. L., and Tortorella, M. D. (1999) Ann. N. Y. Acad. Sci. 878, 105–107.

Tortorella, M. D., Burt, T. C., Pratta, M. A., Abbazsade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., Rockwell, A., Yang, F., Duke, J. S., Solomon, K., George, H., Bruchner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, O. H., Wiswall, B. H., Murphy, K., Hillman, M. C., Jr., Hollis, G. F., Newton, R. C., Magolda, R. L., Trzaskos, J. M., and Arner E. C. (1999) Science 284, 1664–1666.

Abbazsade, I., Liu, R., Yang, F., Rosenfeld, S., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M., Wynn, R., Duke, J. L., George, H. J., Hillman, M. C., Jr., Murphy, K., Wiswall, B. H., Copeland, R. A., Decicco, C. P., Bruchner, R., Nagase, H., Itoh, Y., Newton, R. C., Magolda, R. L., Trzaskos, J. M., Hollis, G. F., Arner, E. C., and Burn, T. C. (1999) J. Biol. Chem. 274, 23443–23450.

Mercuri, F. A., Doee, K. J., Arner, E. C., Pratta, M. A., Last, K., and Fosang, A. J. (1999) J. Biol. Chem. 274, 52887–52895.

Tortorella, M. D., Pratta, M. A., Liu, R. Q., Austin, J., Ross, O. H., Abbazsade, I., Burt, T., and Arner, E. C. (2000) J. Biol. Chem. 275, 18566–18573.