Variations in aggrecan structure modulate its susceptibility to aggrecanases

Peter J. ROUGHLEY*[1], James BARNETT†, Fengrong ZUO† and John S. MORT*

*Shriners Hospital for Children, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6, and †Roche Biosciences, Palo Alto, CA 94304, U.S.A.

Proteoglycan aggregates and purified aggrecan from adult and fetal bovine cartilage and adult and neonatal human cartilage were subjected to *in vitro* degradation by recombinant aggrecanase-1 and aggrecanase-2. The ability of the aggrecanases to cleave within the aggrecan IGD (interglobular domain) and CS2 domain (chondroitin sulphate-rich domain 2) was monitored by SDS/PAGE and immunoblotting. Aggrecanase-2 showed a similar ability to cleave within the IGD of adult and immature aggrecan, whereas aggrecanase-1 was less efficient in cleavage in the IGD of immature aggrecan, for both the bovine and the human substrates. Both aggrecanases showed a similar ability to cleave within the CS2 domain of bovine aggrecan irrespective of age, but showed a much lower ability to cleave within the CS2 domain of human aggrecan. Equivalent results were obtained whether aggrecan was present in isolation or as part of proteoglycan aggregates. When proteoglycan aggregates were used, neither aggrecanase was able to cleave link protein. Thus, for aggrecan cleavage by aggrecanases, variations in cleavage efficiency exist with respect to the species and age of the animal from which the aggrecan is derived and the type of aggrecanase being used.

Key words: ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), age, aggrecan, degradation, metalloprotease, proteoglycan.

INTRODUCTION

All hyaline cartilage is characterized by its turgid nature, which is due in large part to the osmotic properties of the large proteoglycan aggregates that characterize its extracellular matrix. The proteoglycan aggregates are composed of many aggrecan molecules that interact non-covalently with a central filament of hyaluronan, with each interaction being stabilized by association with a link protein [1]. The functional properties of the aggregates depend on the structure and amount of aggrecan, the size of the hyaluronan, and the proportion of link protein. Of particular note is the structure of aggrecan, which can vary between species, with age, and with anatomical site [2].

Aggrecan is composed of a long core protein with several different structural and functional domains [3] (Figure 1). Commencing from its N-terminus, there are two globular domains (G1 and G2) separated by a short IGD (interglobular domain). These are followed by a long extended region, termed the glycosaminoglycan attachment region, to which numerous keratan sulphate and chondroitin sulphate chains are covalently attached. Finally, there is a C-terminal globular domain (G3). The G1 domain is responsible for the interaction of aggrecan with hyaluronan, whereas the function of the other globular domains is less clear. The glycosaminoglycan-attachment region can be divided into three adjacent domains, responsible for the attachment of keratan sulphate (KS domain) or chondroitin sulphate (CS1 and CS2 domains).

Variations in aggrecan structure may arise during both synthesis within the cell and proteolytic processing within the extracellular matrix. Synthetic differences are due mainly to variations in post-translational glycosylation, which may influence both the structure of the glycosaminoglycan chains and the sites at which substitution occurs. Proteolytic processing results in cleavage of the core protein, with retention of the N-terminal product bearing the G1 domain and loss of the C-terminal product from the tissue, as it no longer is able to interact with hyaluronan. It is possible that synthetic variations in glycosaminoglycan substitution may influence the susceptibility of the aggrecan core protein to proteolysis.

Due to its extended structure, the aggrecan core protein is susceptible to cleavage by most proteases that have access to it [4]. However, the proteolytic repertoire of articular cartilage appears to be quite limited. The work of Maniglia and coworkers [5] and Sandy et al. [6] indicated that, in cartilage cultures stimulated with the cytokine interleukin-1, aggrecan is degraded by an activity with the unusual specificity of cleavage following glutamic acid residues. Five cleavage sites in aggrecan were determined (Figure 1): the first is located in the IGD between the G1 and G2 domains, and the other four are in the CS2 domain. The four C-terminal cleavages occur in islands of the core protein lacking chondroitin sulphate substitution sites. Subsequently these cleavages were shown to be due to the action of members of a novel family of metalloproteases termed ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) [7]. These enzymes consist of a metalloprotease unit followed by a disintegrin domain and one or more thrombospondin type 1 motifs. Three of these proteases (ADAMTS-2, -3 and -14) are known to be procollagen N-propeptide cleaving enzymes [8–10]. Another (ADAMTS-13) is able to cleave von Willebrand factor, and its deficiency results in thrombotic thrombocytopaenic purpura [11]. ADAMTS-4 and ADAMTS-5 were isolated from cartilage cultures stimulated with interleukin-1 [12], and since they have been shown to cleavage aggrecan at the previously observed locations (Figure 1), they have been termed aggrecanase-1 and -2 respectively [13,14]. In these enzymes, the thrombospondin motifs have been shown to bind to glycosaminoglycan, and thus appear to assist in substrate recognition [15].

To date, the action of aggrecanases has mostly been studied using bovine nasal cartilage [16] or rat chondrosarcoma aggrecan

Abbreviations used: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; CS1 and CS2 domains, chondroitin sulphate-rich domains of aggrecan; G1, G2, G3, the three globular domains in the aggrecan core protein (N- to C-terminal orientation); IGD, interglobular domain (between aggrecan G1 and G2 domains).

[1] To whom correspondence should be addressed (e-mail proughley@shriners.mcgill.ca).

© 2003 Biochemical Society

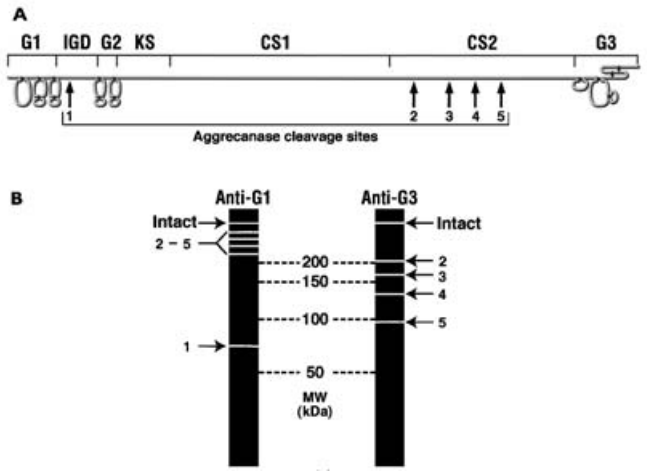

Figure 1 Aggrecanase degradation products of aggrecan

(**A**) Schematic representation of the aggrecan core protein indicating the positions of its constituent domains: G1, G2 and G3 (showing the positions of disulphide bonds), the IGD, KS (the keratan sulphate substitution domain), and CS1 and CS2. Aggrecanase cleavage sites are indicated by numbered arrows. (**B**) Representation of the migration positions of the cleavage products of aggrecan following cleavage by aggrecanase at the positions indicated in (**A**) analysed by SDS/PAGE and immunoblotting using an anti-G1 antibody (left) or anti-G3 antibody (right).

[17] as the substrate. As indicated above, the structure of aggrecan is not constant, but varies with species, age and site of origin. The role of aggrecanases in aggrecan breakdown in health and disease may therefore be modulated by changes in the structure of the substrate. In the present study, using human and bovine cartilage aggrecan prepared from young and adult individuals, it is shown that relative susceptibility to cleavage by aggrecanase is indeed dependent on the source of the substrate.

METHODS

Source of cartilage

Hyaline cartilage was obtained from four sources representing different ages, sites and species. Bovine cartilage was obtained from the nasal septum of a young adult (2 years old) and from the epiphyses of the metacarpal bones of a fetus. Human cartilage was obtained from the distal femurs of an adult (65 years old) and a juvenile (newborn). Cartilage was taken with the permission of the chief pathologist from individuals where the next of kin had given consent for a complete autopsy. The cartilage was stored at -20 °C until used for proteoglycan extraction.

Proteoglycan preparation

Proteoglycan was extracted from finely diced cartilage with 4 M guanidinium chloride in the presence of protease inhibitors at 4 °C for 48 h [18]. Filtered extracts were divided into two equal portions for the preparation of intact proteoglycan aggregates and monomeric aggrecan. For proteoglycan aggregate preparation, the extract was dialysed into 50 mM sodium acetate, pH 6.0, to permit re-association of aggrecan, link protein and hyaluronan, and then adjusted to a density of 1.5 g/ml by the addition of CsCl [19]. For aggrecan preparation, the extract was adjusted directly to a density of 1.5 g/ml by the addition of CsCl while maintaining the guanidine concentration at 4 M [18]. Both extracts were subjected to density-gradient centrifugation at 100000 $\boldsymbol{g}_{av}$ for 48 h at 10 °C. The resulting gradients were fractionated and assayed for density and glycosaminoglycan content. Fractions having a density of >1.55 g/ml were pooled as proteoglycan aggregate preparations from the associative gradients or aggrecan preparations from the dissociative gradients. Both preparations were converted into their sodium salts by dialysis, then freeze-dried and stored at 4 °C until used for protease digestion.

Source of aggrecanase

Full-length ADAMTS-4 and ADAMTS-5 cDNAs were obtained by reverse transcription–PCR based upon the published sequences [13,14]. Briefly, total RNA (1 μg) isolated from human primary synoviocytes was used for cDNA synthesis with a SMART cDNA kit according to the manufacturer's protocol (Clonetech Laboratories, Inc., Palo Alto, CA, U.S.A.). The full-length ADAMTS-4 and ADAMTS-5 cDNAs were then amplified by PCR using primers designed to engineer *Not*I and *Xba*I sites at the 5′ and 3′ ends respectively. The pairs of primers were as follows: 5′-ATGCGGCCGCCTCAATCCTGCAAGCAAGTG-3′ and 5′-CGTCTAGAGCAAGGTCACCACTGT-CAC-3′ for ADAMTS-4, and 5′-ATGCGGCCGCCCTCTGACCTTGGCAGGAGT-3′ and 5′-GCTCTAGAGCATGAGGCAGCAACAGAAGCT-3′ for ADAMTS-5 (restriction sites are underlined). The conditions for PCR reactions were 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. The derived PCR products were digested and ligated into a *Not*I/*Xba*I-digested baculovirus transfer vector. The accuracy of each construct was verified by sequence analysis and restriction digestion. Recombinant baculovirus vectors were produced by co-transfection into Sf9 cells with Baculogold baculovirus DNA (BD Bioscience-Pharmagen). The viruses were plaque-purified and amplified to high-titre seeds.

Sf9 cells were propagated in a bioreactor (B. Braun). At a density of 1.2×10^6 cells/ml, the cultures were infected with recombinant baculovirus that incorporated the gene for either ADAMTS-4 or ADAMTS-5 at a multiplicity of 0.1 plaque-forming unit per cell. After 3 days the cells were pelleted by centrifugation and the supernatant was adjusted to pH 6.7 with concentrated HCl or NaOH. The preparation was then filtered through a 0.45 μm-pore-size capsule filter. A chromatography column packed with SP-Sepharose FF was equilibrated with a buffer consisting of 50 mM Hepes, pH 6.5, 10 mM $CaCl_2$, 0.1 M NaCl and 0.05 % Brij 35. Aggrecanase was absorbed by passing the culture fluid through the column. Elution of the aggrecanase was achieved with a linear NaCl gradient. Fractions from the column that contained aggrecanase activity were pooled. To compare the relative efficacy of the two aggrecanases, the amount of each protease was adjusted to give limit digestion of bovine nasal aggrecan after a 20 h incubation.

Aggrecan digestion

Proteoglycan aggregate or aggrecan (final concentration 2 mg/ml) was digested for different times at 37 °C with aggrecanase-1 or aggrecanase-2 in 50 mM Tris/HCl, pH 8.5, containing 250 mM NaCl and 5 mM $CaCl_2$, conditions reported to give optimal activity of these enzymes [20]. The reactions were terminated by incubation at 70 °C for 5 min; then 5 vol. of ice-cold absolute ethanol was added and the samples were maintained at -20 °C for 1 h. Pellets obtained following centrifugation for 30 min at 16000 $\boldsymbol{g}$ in an Eppendorf centrifuge at 4 °C were allowed to air dry and then were dissolved in 10 mM sodium acetate, pH 6.0, containing 1 mM EDTA and 10 m-units/ml keratanase II (Seikagaku) and incubated at 37 °C for 4 h. Then 0.1 vol. of 1.0 M Tris/acetate, pH 7.3, plus chondroitinase ABC (ICN; final concentration 0.1 unit/ml) were added, and the samples were

© 2003 Biochemical Society

incubated overnight at 37 °C and then analysed by SDS/PAGE. In order to demonstrate limit digestion after overnight (20 h) incubation, a second addition of the same amount of enzyme was made and the samples were incubated for a further 20 h. All experiments were performed at least twice using two different batches of each recombinant protease. Representative data showing blots from the same digest are depicted.

SDS/PAGE and immunoblotting

Samples were run under reducing conditions on Novex 4–12 % (w/v) gradient NuPage Bis-Tris gels (Invitrogen) and electroblotted to nitrocellulose membranes (Bio-Rad) as recommended by the manufacturer using a Novex transfer apparatus. The membranes were then blocked overnight in 3 % (w/v) BSA in Tris-buffered saline (10 mM Tris/HCl, pH 8.0, 150 mM NaCl) containing 0.05 % Tween 20. Immunodetection of the aggrecan degradation products was performed utilizing the antibodies described below and an alkaline phosphatase-conjugated goat anti-(rabbit IgG) or anti-(mouse IgG) antibody (Promega). Colour was developed using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [21]. All washing of the blots was carried out in Tris-buffered saline containing 0.05 % Tween 20. Anti-peptide antibodies specific for the G1 and the G2 domains of human and bovine aggrecan, and the G3 domain of human aggrecan, and anti-neoepitope antibodies specific for the aggrecanase cleavage products of human and bovine aggrecan, have been described previously [22]. An anti-peptide antibody to the G3 domain of bovine aggrecan was prepared by immunization of a rabbit with a mixture of ovalbumin conjugates of the peptides GWTKFQGHCYRHFPDR, TWVDAESQCRKQQSHLSS and CQPSGHWEEPRIT. Conjugation, immunization and characterization of the resulting antisera were carried out as described previously [23]. The mouse monoclonal antibody 8A4 to link protein [24] was a gift from Dr. B. Caterson (University of Wales, Cardiff, U.K.).

RESULTS

Aggrecan from adult or juvenile bovine or adult or juvenile human cartilage was treated with aggrecanase-1 or aggrecanase-2 in order to determine how cleavage by the different aggrecanases might be influenced by differences in aggrecan structure. The ability of the aggrecanases to cleave at different sites in the aggrecan molecules was studied by immunoblotting of digests. In order to analyse cleavage within the IGD of the aggrecan core proteins, immunoblots were developed using an antiserum raised against the G1 domain (Figures 2 and 3).

Treatment of adult bovine nasal cartilage aggrecan with aggrecanase-1 or aggrecanase-2 resulted in a similar profile for G1 generation (Figure 2). In the absence of aggrecanase treatment, no free G1 domain was detected in the aggrecan preparation, but upon exposure to either aggrecanase two components with molecular masses of approx. 90 and 80 kDa were detected. The proportions of the two components remained constant with time, and both components showed the presence of the same C-terminus (. . .NITEGE) when analysed using an anti-neoepitope antibody to the aggrecanase cleavage site in the bovine aggrecan IGD (results not shown). Thus it is likely that the smaller component is not a proteolytic degradation product of the larger component, but differs in its post-translational glycosylation.

Treatment of fetal bovine epiphyseal cartilage aggrecan with the two aggrecanases also resulted in the generation of two G1 components that were not detected in the untreated aggrecan

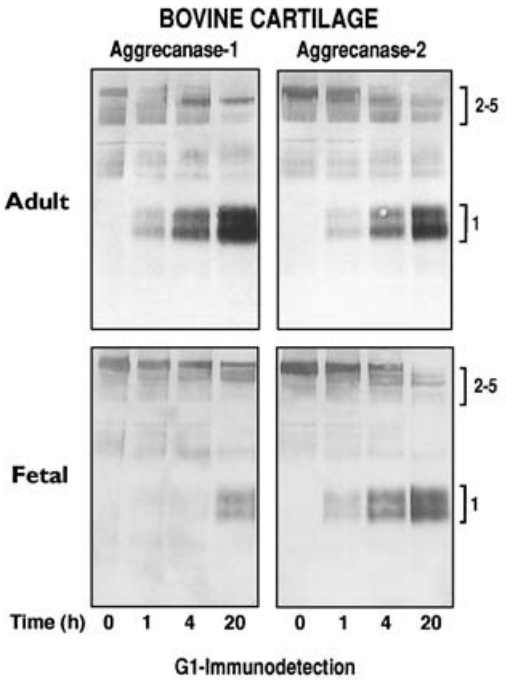

Figure 2 Production of G1-containing fragments from bovine cartilage aggrecan

Aggrecan from adult or fetal bovine cartilage was subjected to degradation by aggrecanase-1 or aggrecanase-2 for periods of 1, 4 or 20 h. Undigested controls (0 h) and the three digests were analysed by SDS/PAGE and subsequent immunoblotting using an antibody recognizing the G1 domain. The expected migration positions of G1-containing products arising from cleavage within the IGD (site 1) or the CS2 domain (sites 2–5) are indicated (see Figure 1).

(Figure 2). These G1 components were of similar molecular size to those released from the adult nasal cartilage aggrecan. However, in contrast with the adult nasal cartilage aggrecan, free G1 generation by aggrecanase-2 was much more pronounced than that by aggrecanase-1. Both the fetal and adult bovine aggrecans also showed evidence of cleavage within the aggrecan CS2 domain, resulting in new components being generated near the migration position of the intact aggrecan. No components of intermediate size between those compatible with CS2 and IGD cleavage were detected, even though products in such a size range were detected in the untreated aggrecan preparations.

When adult human articular cartilage aggrecan was treated with the two aggrecanases, similar profiles for G1 generation were obtained (Figure 3) as were observed with adult bovine aggrecan. However, in contrast with the bovine aggrecan G1, the human aggrecan G1 appeared as only a single component with a molecular mass of approx. 75 kDa. Small amounts of this G1 component were detected in the untreated adult human aggrecan, together with a second G1 component of smaller molecular size. Analysis of the immunoblots using an anti-neoepitope antibody to the new C-terminus generated by matrix metalloprotease-mediated cleavage within the human aggrecan IGD revealed a product identical in size with this smaller G1 component (results not shown). The untreated aggrecan preparation also contained several components of a size intermediate between those of intact aggrecan and the free G1 domains, but none of these components were altered in abundance or size by aggrecanase treatment. These products are expected to be generated by cleavage between the aggrecan CS2 and IGD domains.

© 2003 Biochemical Society

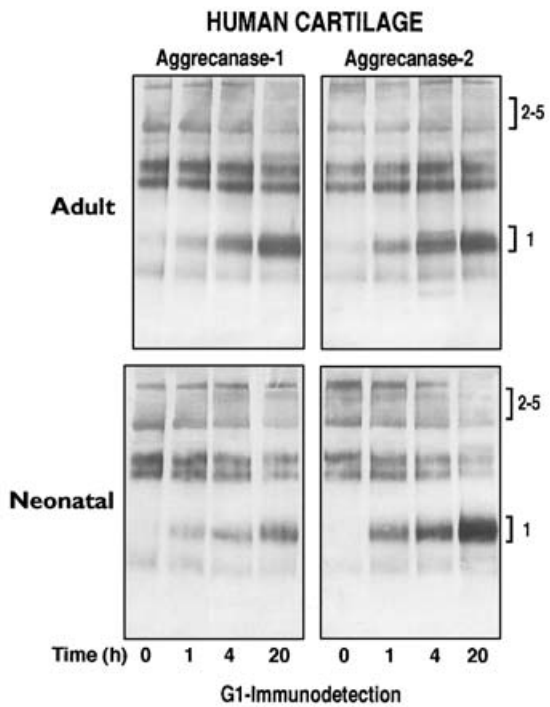

Figure 3 Production of G1-containing fragments from human cartilage aggrecan

Aggrecan from adult or neonatal human cartilage was subjected to degradation by aggrecanase-1 or aggrecanase-2 for periods of 1, 4 or 20 h. Undigested controls (0 h) and the three digests were analysed by SDS/PAGE and subsequent immunoblotting using an antibody recognizing the G1 domain. The expected migration positions of G1-containing products arising from cleavage within the IGD (site 1) or the CS2 domain (sites 2–5) are indicated (see Figure 1).

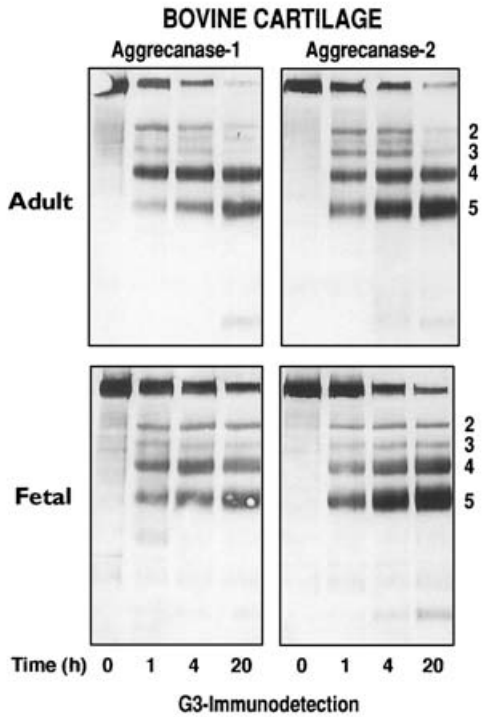

Figure 4 Production of G3-containing fragments from bovine cartilage aggrecan

Aggrecan from adult or fetal cartilage was subjected to degradation by aggrecanase-1 or aggrecanase-2 for periods of 1, 4 or 20 h. Undigested controls (0 h) and the three digests were analysed by SDS/PAGE and subsequent immunoblotting using an antibody recognizing the G3 domain. The expected migration positions of G3-containing products arising from cleavage within the CS2 domain (sites 2–5) are indicated (see Figure 1).

Treatment of juvenile human cartilage aggrecan with the two aggrecanases also gave rise to a single G1 component of the same molecular size as that obtained upon treatment of the adult human aggrecan (Figure 3). However, G1 generation was more pronounced following treatment with aggrecanase-2 than with aggrecanase-1, as was observed with the fetal bovine aggrecan. In all the systems studied, the amount of G1 released at the end of the incubation period could not be increased by exposure of the digest to additional aggrecanase. Thus, in the juvenile preparation, a proportion of the aggrecan that is accessible to cleavage by aggrecanase-2 is not accessible to cleavage by aggrecanase-1. As with that from the adult, the juvenile human aggrecan preparation also contained several degradation products of the intact aggrecan that cannot be generated by aggrecanase treatment. However, in the case of the juvenile it is possible that some of these partial degradation products may be cleaved by the aggrecanases to yield free G1 domains.

Analysis of degradation products by immunoblotting with the anti-(G1 domain) antibody is not suited to studying cleavage within the aggrecan CS2 domain, as the products do not resolve well from the intact aggrecan or from each other on the electrophoresis system being used. To address the issue of CS2 domain cleavage, analogous blots were probed with anti-(G3 domain) antibodies (Figures 4 and 5).

Treatment of adult bovine nasal cartilage aggrecan with either aggrecanase-1 or aggrecanase-2 gave similar results, with two major products resulting from cleavage at the most C-terminal sites (sites 4 and 5; see Figure 1) accumulating with time (Figure 4). Exposure to additional aggrecanase did not alter this pattern and could not accomplish total cleavage at the most C-terminal site. Thus some aggrecan molecules exhibit resistance to aggrecan cleavage at this site. At earlier time points, products corresponding to the more N-terminal aggrecanase cleavage sites (sites 2 and 3; see Figure 1) were detected, together with a product that appears to be of intermediate size. The identity of the known cleavage sites was confirmed by the use of N-terminal anti-neoepitope antibodies, and all the major G3-containing products (with the exception of that between sites 2 and 3) had the expected N-terminal recognition. No products representative of aggrecanase-mediated cleavage in the CS2 domain were detected in the initial aggrecan preparation.

Treatment of the fetal bovine epiphyseal cartilage aggrecan with the two aggrecanases also resulted in products representing cleavage at all four known aggrecanase cleavage sites in the CS2 domain (Figure 4). Once again, cleavage at sites 4 and 5 predominated, and additional protease could not convert all the products to a single size. In contrast with the adult, products formed by cleavage at sites 2 and 3 did persist after prolonged enzyme exposure, and the product of intermediate size was not detected. In addition, aggrecanase-1 appeared to be less efficient than aggrecanase-2 at aggrecan cleavage in the fetal compared with adult bovine aggrecan.

Identical profiles were obtained when adult human articular cartilage aggrecan was treated with the two aggrecanases (Figure 5). In contrast with the bovine system, there was little evidence for cleavage within the CS2 domain, and cleavage at only

© 2003 Biochemical Society

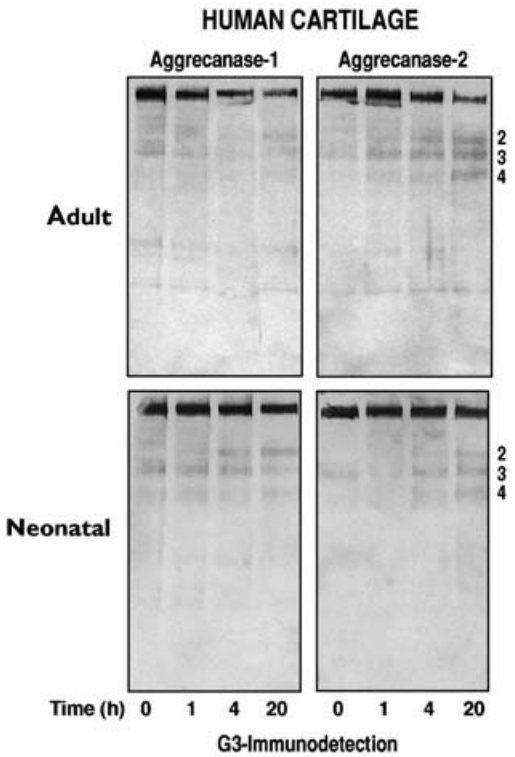

Figure 5 Production of G3-containing fragments from human cartilage aggrecan

Aggrecan from adult or neonatal cartilage was subjected to degradation by aggrecanase-1 or aggrecanase-2 for periods of 1, 4 or 20 h. Undigested controls (0 h) and the three digests were analysed by SDS/PAGE and subsequent immunoblotting using an antibody recognizing the G3 domain. The expected migration positions of G3-containing products arising from cleavage within the CS2 domain (sites 2–4) are indicated (see Figure 1).

three sites was apparent. The use of anti-neoepitope antibodies confirmed that the three G3-containing products corresponded to cleavage at sites 2, 3 and 4 in the CS2 domain, and that products corresponding to cleavage at site 5 (see Figure 1) were not detected. Once generated, there appeared to be little evidence for conversion of larger products into smaller products with time.

Treatment of the juvenile human epiphyseal cartilage aggrecan with the two aggrecanases also resulted in only minimal cleavage in the CS2 domain (Figure 5), and resulted in a product distribution similar to that observed from the adult human aggrecan. However, in contrast with the adult, it appeared that aggrecanase-1 was less efficient at cleavage within the juvenile aggrecan CS2 domain than aggrecanase-2, in a similar manner to the situation in the bovine system.

Proteoglycan aggregate preparations from the same four cartilage sources described above were also subjected to degradation by aggrecanase-1 and aggrecanase-2 in an identical manner with that of the purified aggrecan. The degree and pattern of cleavage at the five known aggrecanase sites was the same irrespective of whether aggrecan was present in a monomeric or aggregated form (results not shown). In the case of the proteoglycan aggregates, immunoblotting was also performed using an anti-(link protein) antibody (Figure 6). It was apparent that neither bovine nor human link protein was susceptible to cleavage by either aggrecanase-1 or aggrecanase-2.

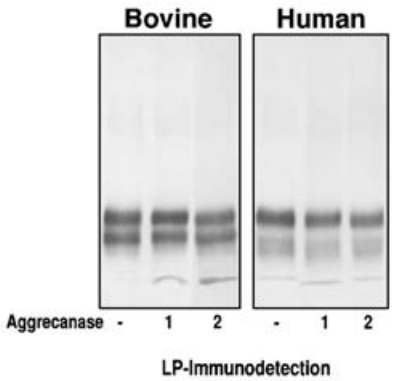

Figure 6 Resistance of link protein to aggrecanase action

Proteoglycan aggregates from fetal bovine epiphyseal cartilage or neonatal human articular cartilage were subjected to degradation by aggrecanase-1 (1) or aggrecanase-2 (2) for 20 h. Undigested (—) and digested (1 or 2) samples were analysed by SDS/PAGE and subsequent immunoblotting using an antibody specific for link protein (LP).

DISCUSSION

This work demonstrates that not all aggrecan molecules are created equal in their susceptibility to cleavage by aggrecanase, and that aggrecanase-1 and aggrecanase-2 do not treat all aggrecan molecules equally. With the different bovine and human aggrecan preparations used, it is apparent that cleavage within the aggrecan CS2 domain is a much more favoured event in the bovine than in the human, whereas cleavage within the IGD occurs well in both species with aggrecanase-2, but is less efficient with aggrecanase-1, particularly in the bovine fetus. These variations presumably reflect differences in both aggrecan and aggrecanase structure.

The variation in IGD cleavage between aggrecan from young and old cartilage probably relates to differences in keratan sulphate substitution within this domain. Keratan sulphate substitution close to the aggrecanase IGD cleavage site has been reported for both pig and bovine aggrecan [25,26]. Such substitution may be either O-linked to threonine or N-linked to asparagine, and of particular note is the substitution of the asparagine and threonine residues in the sequence ....NITEGE... that forms the N-terminal side of the aggrecanase cleavage site. During juvenile development, the composition of the keratan sulphate chains changes and their lengths increase [27,28]. Initially, it had been suggested that keratan sulphate substitution might impede protease access to the cleavage site and so provide protection from proteolysis. This being the case, it might be predicted that protection would be greatest in the adult aggrecan, with the longer keratan sulphate chains. This, however, is the opposite to what is observed in the present study. It is now appreciated that the presence of keratan sulphate can promote IGD cleavage in intact aggrecan by aggrecanase-1, and that keratanase treatment to remove the keratan sulphate chains represses cleavage [29]. Cleavage by keratanase results in the asparagine and threonine residues being substituted with short N-linked and O-linked oligosaccharides, which mimics their substitution in the fetus and hence explains the less prominent IGD cleavage by aggrecanase-1. However, it is apparent from studies using a recombinant IGD that cleavage by aggrecanase-1 can occur even in the absence of keratan sulphate [30,31]. The results of the present work would also suggest that the presence of keratan sulphate substitution has a lower impact on aggrecanase-2 than aggrecanase-1.

© 2003 Biochemical Society

It has been reported that, in both bovine and rat aggrecan, cleavage within the CS2 domain occurs preferentially relative to cleavage within the IGD [16,17,20,32]. In the present work, such preferential CS2 cleavage did not appear to occur in human aggrecan from either juvenile or adult. The reason for this difference is not obvious, as the amino acid sequence surrounding the four CS2 aggrecanase cleavage sites shows little species variation that might be expected to influence recognition by aggrecanase. It is possible that species differences in glycosylation might be involved. It is accepted that chondroitin sulphate substitution is essential for efficient aggrecanase-mediated cleavage within the CS2 domain [15,20], and while there is little variation in the presence of potential chondroitin sulphate substitution sites between species, it is possible that these sites could show species variations in their use. It is also possible that different chondroitin sulphate structures are not equal in their interaction with the aggrecanases. Of particular note is the higher degree of 6-sulphation relative to 4-sulphation that occurs in the human [18,33]. Finally, it is noteworthy that threonine residues that could act as potential attachment sites for O-linked oligosaccharides or keratan sulphate occur close to each of the four aggrecanase CS2 cleavage sites. Species variations in their substitution could also influence the susceptibility to cleavage by aggrecanase.

In bovine aggrecan, it has been reported that cleavage at site 3 within the CS2 domain is a preferred event, with subsequent cleavage at the other sites [16]. If cleavage at sites 4 and 5 occurred to completion, one would expect that, at the limit of digestion, the only G3-containing degradation product would be that bearing an N-terminal sequence corresponding to cleavage at site 5 (see Figure 1). This was not, however, the case, as G3-containing products arising from cleavage at either site 4 or site 5 were both prominent. This implies that cleavage at site 5 does not occur in at least some of the aggrecan molecules following cleavage at site 4. Once again, it is possible that variation in glycosylation could contribute to this phenomenon, as there is no reason why all aggrecan molecules need to be glycosylated identically in the same cartilage. It is also possible that aggrecan cleavage is not a progressive process, and that cleavage may occur at either site 4 or site 5 following site 3, but that once it has occurred at site 4 it can then no longer occur at site 5.

Differences between aggrecanase-1 and aggrecanase-2 have been reported with regard to cleavage within the CS2 domain of bovine cartilage aggrecan. Aggrecanase-2 appears to cleave more slowly than aggrecanase-1, but also acts at an additional cleavage site between sites 2 and 3 [32]. The occurrence of such a site was observed in the present work for bovine aggrecan from mature nasal cartilage, but not for that from fetal epiphyseal cartilage. The present work also showed that, for all four aggrecans studied, the profile of aggrecanase-mediated cleavage occurred similarly in both the free monomer and the proteoglycan aggregate. Thus the close packing of G1 domains along hyaluronan and the proximity of link protein do not appear to impede access to the IGD by either aggrecanase-1 or aggrecanase-2. Incubation of the proteoglycan aggregates also revealed that neither human nor bovine link protein could act as a substrate for cleavage by aggrecanase. Others have shown that proteins such as collagens, casein and fibronectin are not substrates for aggrecanase cleavage [32], but that proteoglycans related to aggrecan, such as versican and brevican [34,35], can be cleaved by aggrecanase. As present it is not clear whether this is due to the absence of an appropriate amino acid sequence or glycosaminoglycan substitution in the non-proteoglycans.

It has recently been reported that ADAMTS-1 can act as an aggrecanase and cleave at the same IGD and CS2 sites as aggrecanase-1 and aggrecanase-2 [36]. In contrast with aggrecanase-1 and aggrecanase-2 in the present study, ADAMTS-1 did appear to cleave efficiently within the CS2 domain of human aggrecan, and cleavage at site 5 was apparent. As ADAMTS-1 mRNA expression has been identified in human articular cartilage [37], the properties of ADAMTS-1 could ideally complement those of the other aggrecanases during the *in vivo* proteolysis of aggrecan. Evidence for cleavage at site 5, *in vivo*, has been obtained by analysis of aggrecan fragments in synovial fluid [38], although the protease responsible was unresolved.

It is apparent from the present work that aggrecan degradation products possessing the G3 domain are not prominent in aggrecan preparations, in contrast with those possessing the G1 domain. It is unlikely that this is an artefact of purification by CsCl density gradient centrifugation, as all G3-containing products should possess sufficient chondroitin sulphate to ensure their sedimentation to high density. Instead, it is likely that this reflects loss of the G3-containing products from the tissue *in vivo* following the proteolytic processing of aggrecan. While fragments bearing the G1 domain remain localized via their interaction with hyaluronan, those bearing the G3 domain appear to have no equivalent anchor. The aggrecan G3 domain has been reported to interact with several matrix molecules [39], but none of these interactions alone appear to be sufficient for matrix retention.

It is also apparent that, in the human proteoglycan preparations, there are G1-containing components of larger size than free G1 domains that are not cleaved by aggrecanase treatment (Figure 3). Use of an anti-(G2 domain) antibody showed that all of these components possessed both G1 and G2 domains (results not shown) and hence contained an intact IGD. Presumably the inability of aggrecanase to cleave within the IGD reflects a structure incompatible with cleavage, again possibly due to glycosylation variants. It is likely that these products were synthesized earlier in life than the more intact material in the preparations, and have survived because of their resistance to cleavage by aggrecanase. Such resistance in the adult appears to be similar for aggrecanase-1 and aggrecanase-2, and hence may not reflect the same origin as the decreased affinity of the juvenile aggrecan for cleavage by aggrecanase-1 in the IGD. This resistance to IGD cleavage is certainly not a property of all human aggrecan IGD structures, as it has been shown that aggrecanase-1 can cleave between the G1 and G2 domains in a recombinant protein [40]. However, it is not clear how the glycosylation of this protein relates to that present *in vivo*, and whether this may influence susceptibility to aggrecanase.

In addition to their role in normal cartilage growth and remodelling [41], aggrecanases are also believed to mediate proteoglycan destruction in the arthritic joint [42,43]. It is clear that differences in susceptibility to these metalloproteases could have important implications for disease progress, and for the future of aggrecanase inhibitors as a potential preventative treatment for osteoarthritis.

This work was supported by the Canadian Institutes of Health Research, The Arthritis Society of Canada and the Shriners of North America. The technical assistance of Robert White is gratefully acknowledged. We thank Elisa de Miguel for peptide synthesis and Guylaine Bédard for preparing the figures.

REFERENCES

1 Hascall, V. C. (1988) Proteoglycans: the chondroitin sulfate/keratan sulfate proteoglycans of cartilage. ISI Atlas Sci. Biochem. **1**, 189–198

2 Roughley, P. J. and Mort, J. S. (1986) Ageing and the aggregating proteoglycans of human articular cartilage. Clin. Sci. **71**, 337–344

3 Watanabe, H., Yamada, Y. and Kimata, K. (1998) Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. J. Biochem. (Tokyo) **124**, 687–693

© 2003 Biochemical Society

4 Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1992) The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J. Biol. Chem. **267**, 19470–19474

5 Loulakis, P., Shrikhande, A., Davis, G. and Maniglia, C. A. (1992) N-Terminal sequence of proteoglycan fragments isolated from medium of interleukin-1-treated articular-cartilage cultures. Biochem. J. **284**, 589–593

6 Sandy, J. D., Neame, P. J., Boynton, R. E. and Flannery, C. R. (1991) Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J. Biol. Chem. **266**, 8683–8685

7 Tang, B. L. (2001) ADAMTS: a novel family of extracellular matrix proteases. Int. J. Biochem. Cell Biol. **33**, 33–44

8 Colige, A., Li, S. W., Sieron, A. L., Nusgens, B. V., Prockop, D. J. and Lapière, C. M. (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. Proc. Natl. Acad. Sci.U.S.A. **94**, 2374–2379

9 Fernandes, R. J., Hirohata, S., Engle, J. M., Colige, A., Cohn, D. H., Eyre, D. R. and Apte, S. S. (2001) Procollagen II amino propeptide processing by ADAMTS-3: insights on dermatosparaxis. J. Biol. Chem. **276**, 31502–31509

10 Colige, A., Vandenberghe, I., Thiry, M., Lambert, C. A., Van Beeumen, J., Li, S. W., Prockop, D. J., Lapière, C. M. and Nusgens, B. V. (2002) Cloning and characterization of ADAMTS-14, a novel ADAMTS displaying high homology with ADAMTS-2 and ADAMTS-3. J. Biol. Chem. **277**, 5756–5766

11 Levy, G. G., Nichols, W. C., Lian, E. C., Foroud, T., McClintick, J. N., McGee, B. M., Yang, A. Y., Siemieniak, D. R., Stark, K. R., Gruppo, R. et al. (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature (London) **413**, 488–494

12 Arner, E. C., Pratta, M. A., Trzaskos, J. M., Decicco, C. P. and Tortorella, M. D. (1999) Generation and characterization of aggrecanase: a soluble, cartilage-derived aggrecan-degrading activity. J. Biol. Chem. **274**, 6594–6601

13 Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R. et al. (1999) Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science **284**, 1664–1666

14 Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M. et al. (1999) Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J. Biol. Chem. **274**, 23443–23450

15 Tortorella, M. D., Pratta, M., Liu, R. Q., Abbaszade, I., Ross, H., Burn, T. and Arner, E. (2000) The thrombospondin motif of aggrecanase-1 (ADAMTS-4) is critical for aggrecan substrate recognition and cleavage. J. Biol. Chem. **275**, 25791–25797

16 Tortorella, M. D., Pratta, M., Liu, R. Q., Austin, J., Ross, O. H., Abbaszade, I., Burn, T. and Arner, E. (2000) Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4). J. Biol. Chem. **275**, 18566–18573

17 Sandy, J. D., Thompson, V., Doege, K. and Verscharen, C. (2000) The intermediates of aggrecanase-dependent cleavage of aggrecan in rat chondrosarcoma cells treated with interleukin-1. Biochem. J. **351**, 161–166

18 Roughley, P. J. and White, R. J. (1980) Age-related changes in the structure of the proteoglycan subunits from human articular cartilage. J. Biol. Chem. **255**, 217–224

19 Bayliss, M. T. and Roughley, P. J. (1985) The properties of proteoglycan prepared from human articular cartilage by using associative caesium chloride gradients of high and low starting densities. Biochem. J. **232**, 111–117

20 Sugimoto, K., Takahashi, M., Yamamoto, Y., Shimada, K. and Tanzawa, K. (1999) Identification of aggrecanase activity in medium of cartilage culture. J. Biochem. (Tokyo) **126**, 449–455

21 Sztrolovics, R., Alini, M., Roughley, P. J. and Mort, J. S. (1997) Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem. J. **326**, 235–241

22 Sztrolovics, R., White, R. J., Roughley, P. J. and Mort, J. S. (2002) The mechanism of aggrecan release from cartilage differs with tissue origin and the agent used to stimulate catabolism. Biochem. J. **362**, 465–472

23 Mach, L., Schwihla, H., Stüwe, K., Rowan, A. D., Mort, J. S. and Glössl, J. (1993) Activation of procathepsin B in human hepatoma cells: the conversion to the mature enzyme relies on the action of cathepsin B itself. Biochem. J. **293**, 437–442

24 Caterson, B., Christner, J. E., Baker, J. R. and Couchman, J. R. (1985) Production and characterization of monoclonal antibodies directed against connective tissue proteoglycan. Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 386–393

25 Barry, F. P., Gaw, J. U., Young, C. N. and Neame, P. J. (1992) Hyaluronan-binding region of aggrecan from pig laryngeal cartilage: amino acid sequence, analysis of N-linked oligosaccharides and locations of the keratan sulphate. Biochem. J. **286**, 761–769

26 Barry, F. P., Rosenberg, L. C., Gaw, J. U., Koob, T. J. and Neame, P. J. (1995) N- and O-linked keratan sulfate on the hyaluronan binding region of aggrecan from mature and immature bovine cartilage. J. Biol. Chem. **270**, 20516–20524

27 Brown, G. M., Huckerby, T. N., Bayliss, M. T. and Nieduszynski, I. A. (1998) Human aggrecan keratan sulfate undergoes structural changes during adolescent development. J. Biol. Chem. **273**, 26408–26414

28 Plaas, A. H. K., West, L. A. and Midura, R. J. (2001) Keratan sulfate disaccharide composition determined by FACE analysis of keratanase II and endo-β-galactosidase digestion products. Glycobiology **11**, 779–790

29 Pratta, M. A., Tortorella, M. D. and Arner, E. C. (2000) Age-related changes in aggrecan glycosylation affect cleavage by aggrecanase. J. Biol. Chem. **275**, 39096–39102

30 Hughes, C. E., Büttner, F. H., Eidenmüller, B., Caterson, B. and Bartnik, E. (1997) Utilization of a recombinant substrate rAgg1 to study the biochemical properties of aggrecanase in cell culture systems. J. Biol. Chem. **272**, 20269–20274

31 Mercuri, F. A., Doege, K. J., Arner, E. C., Pratta, M. A., Last, K. and Fosang, A. J. (1999) Recombinant human aggrecan G1-G2 exhibits native binding properties and substrate specificity for matrix metalloproteinases and aggrecanase. J. Biol. Chem. **274**, 32387–32395

32 Tortorella, M. D., Liu, R. Q., Burn, T., Newton, R. C. and Arner, E. (2002) Characterization of human aggrecanase 2 (ADAM-TS5): substrate specificity studies and comparison with aggrecanase 1 (ADAM-TS4). Matrix Biol. **21**, 499–511

33 Murata, K. and Bjelle, A. O. (1979) Age-dependent constitution of chondroitin sulfate isomers in cartilage proteoglycans under associative conditions. J. Biochem. (Tokyo) **86**, 371–376

34 Sandy, J. D., Westling, J., Kenagy, R. D., Iruela-Arispe, M. L., Verscharen, C., Rodrique-Mazaneque, J., Zimmermann, D. R., Lemire, J. M., Fischer, J. W., Wight, T. N. and Clowes, A. W. (2001) Versican V1 proteolysis in human aorta *in vivo* occurs at the Glu^{441}-Ala^{442} bond, a site which is cleaved by recombinant ADAMTS-1 and ADAMTS-4. J. Biol. Chem. **276**, 13372–13378

35 Nakamura, H., Fujii, Y., Inoki, I., Sugimoto, K., Tanzawa, K., Matsuki, H., Miura, R., Yamaguchi, Y. and Okada, Y. (2000) Brevican is degraded by matrix metalloproteinases and aggrecanase-1 (ADAMTS4) at different sites. J. Biol. Chem. **275**, 38885–38890

36 Rodríguez-Manzaneque, J. C., Westling, J., Thai, S. N. M., Luque, A., Knäuper, V., Murphy, G., Sandy, J. D. and Iruela-Arispe, M. L. (2002) ADAMTS1 cleaves aggrecan at multiple sites and is differentially inhibited by metalloproteinase inhibitors. Biochem. Biophys. Res. Commun. **293**, 501–508

37 Flannery, C. R., Little, C. B., Hughes, C. E. and Caterson, B. (1999) Expression of ADAMTS homologues in articular cartilage. Biochem. Biophys. Res. Commun. **260**, 318–322

38 Sandy, J. D. and Verscharen, C. (2001) Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing *in vivo*. Biochem. J. **358**, 615–626

39 Olin, A. I., Mörgelin, M., Sasaki, T., Timpl, R., Heinegård, D. and Aspberg, A. (2001) The proteoglycans aggrecan and versican form networks with fibulin-2 through their lectin domain binding. J. Biol. Chem. **276**, 1253–1261

40 Westling, J., Fosang, A. J., Last, K., Thompson, V. P., Tomkinson, K. N., Hebert, T., McDonagh, T., Collins-Racie, L. A., LaVallie, E. R., Morris, E. A. and Sandy, J. D. (2002) ADAMTS4 cleaves at the aggrecanase site (Glu^{373}-Ala^{374}) and secondarily at the matrix metalloproteinase site (Asn^{341}-Phe^{342}) in the aggrecan interglobular domain. J. Biol. Chem. **277**, 16059–16066

41 Lee, E. R., Lamplugh, L., Davoli, M. A., Beauchemin, A., Chan, K., Mort, J. S. and Leblond, C. P. (2001) Enzymes active in the areas undergoing cartilage resorption during the development of the secondary ossification center in the tibiae of rats aged 0–21 days: I. Two groups of proteinases cleave the core protein of aggrecan. Dev. Dyn. **222**, 52–70

42 Lohmander, L. S., Neame, P. J. and Sandy, J. D. (1993) The structure of aggrecan fragments in human synovial fluid: evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury and osteoarthritis. Arthritis Rheum. **36**, 1214–1222

43 Malfait, A. M., Liu, R. Q., Ijiri, K., Komiya, S. and Tortorella, M. D. (2002) Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. J. Biol. Chem. **277**, 22201–22208

Received 23 April 2003/18 June 2003; accepted 15 July 2003
Published as BJ Immediate Publication 15 July 2003, DOI 10.1042/BJ20030609

© 2003 Biochemical Society