Mutations in the interglobular domain of aggrecan alters matrix metalloproteinase and aggrecanase cleavage patterns: Evidence that matrix metalloproteinase cleavage interferes with aggrecanase activity

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Running Title: MMP and aggreganase cleavage-site mutants in recombinant G1-G2
Abstract
We have expressed G1-G2 mutants with amino acid changes at the DIPEN$_{341}$ $\downarrow$ F$_{342}$FGVG and ITEGE$_{373}$ $\downarrow$ A$_{374}$RGSV cleavage sites, in order to investigate the relationship between matrix metalloproteinase (MMP) and aggrecanase activities in the interglobular domain (IGD) of aggrecan. The mutation DIPEN$_{341}$ to DIGSA$_{341}$ partially blocked cleavage by MMP-13 and MMP-8 at the MMP site, while the mutation F$_{342}$FGVG to G$_{342}$TRVG completely blocked cleavage at this site by MMP-1, -2, -3, -7, -8, -9, -13, -14. Each of the MMP cleavage site mutants, including a four amino acid deletion mutant lacking residues ENFF$_{343}$, were efficiently cleaved by aggrecanase suggesting that the primary sequence at the MMP site had no effect on aggrecanase activity in the IGD. The mutation A$_{374}$RGSV to N$_{374}$VYSV completely blocked cleavage at the aggrecanase site by aggrecanase, MMP-8 and atrolysin C but had no effect on the ability of MMP-8 and MMP-13 to cleave at the N$_{341}$ $\downarrow$ F bond. Susceptibility to atrolysin C cleavage at the MMP site was conferred in the DIGSA$_{341}$ mutant but absent in the wildtype, G$_{342}$TRVG, N$_{374}$VYSV and deletion mutants. To further explore the relationship between MMP and aggrecanase activities, sequential digest experiments were done in which MMP degradation products were subsequently digested with aggrecanase, and vice versa. Aggrecanase-derived G1 domains with ITEGE$_{373}$ C-termini were viable substrates for MMPs, however MMP-derived G2 fragments were resistant to cleavage by aggrecanase. A 10mer peptide FVDIPENFFG, which is a substrate analogue for the MMP cleavage site, inhibited aggrecanase cleavage at the E$_{373}$ $\downarrow$ A$_{374}$ bond. This study demonstrates that MMPs and aggrecanase have unique substrate recognition in the IGD of aggrecan and suggests that sequences at the C-terminus of the DIPEN$_{341}$ G1 domain may be important for regulating aggrecanase cleavage.
Introduction
The large proteoglycan aggrecan is removed from cartilage by the action of at least two families of zinc-dependent metalloenzymes, the matrix metalloproteinases (MMPs)\(^1\) and the aggrecanases. The MMPs are a family of matrix degrading enzymes, comprising collagenases, gelatinases, stromelysins and the membrane-bound MT-MMPs (reviewed in 1,2). Aggrecanase-1 (3) and -2 (4,5) that exhibit the characteristic activity of E\(_{373}\) ↓ A\(_{374}\) hydrolysis in the IGD have recently been identified as members of the ADAMTS family (6) of proteinases containing disintegrin, metalloproteinase and thrombospondin motifs. Identification of MMP (7-12) and aggrecanase (13-15) cleavage sites in the aggrecan IGD has enabled monitoring of MMP activity via detection of DIPEN\(_{341}\) and F\(_{342}\)FGVG terminal sequences, and aggrecanase activity via detection of ITEGE\(_{373}\) and A\(_{374}\)RGSV sequences, present on digestion products. Both MMP and aggrecanase derived fragments have been detected \(\text{in vivo}\) by amino acid sequence analysis (8,16,17) and more recently by the use of neoepitope antibodies (18-28).

Aggrecanase is the major mediator of aggrecanolysis \(\text{in vitro}\) (29-32), however MMPs are also active and the relationship between these activities is not understood. Three potential models for cleavage in the IGD have been proposed (33,34). In the first model, aggrecan is cleaved initially by aggrecanase, and the G1 fragments remaining in the tissue are subsequently cleaved by MMPs. Model 1 therefore generates aggrecanase and MMP G1 fragments, and a 32 amino acid F\(_{342}\) - E\(_{373}\) fragment, but fails to account for the presence of F\(_{342}\)FGVG fragments in which the aggrecanase site remains intact (19). In the second model, aggrecan is cleaved initially by MMPs, and the released fragment containing the F\(_{342}\)FGVG neoepitope is subsequently cleaved by aggrecanase. Model 2 generates both aggrecanase (with A\(_{374}\)RGSV N-terminus) and MMP (with F\(_{342}\)FGVG N-terminus) fragments, and the 32 amino acid F\(_{342}\) - E\(_{373}\) fragment, but fails to account for the presence of aggrecanase G1 fragments present in human (21,22), bovine (35,29), pig (29), rat (35) and mouse (23,26) cartilage. Model 3, which proposes that aggrecanase and MMP activities are independent, is favoured by us (19) and others (21,22). We favour this model because in studies investigating the validity of each model (34)(see companion paper) we have observed that both ITEGE\(_{373}\) G1 domains and large F\(_{342}\)FGVG fragments are generated during IL-1\(\alpha\) treatment of pig articular cartilage \(\text{in vitro}\). Since the ITEGE\(_{373}\) G1 domain and F\(_{342}\)FGVG fragments cannot be derived from the one aggrecan molecule, we concluded that in pig cartilage stimulated with IL-1\(\alpha\), MMP and aggrecanase activities were mutually exclusive (36).

The aim of this study was to resolve the mechanism by which MMP and aggrecanase activities could be mutually exclusive. We asked whether mutations at the MMP cleavage site would affect cleavage by aggrecanase or vice versa, and we asked whether the products of one activity were viable substrates

\(^1\) Abbreviations
ADAM, A Disintegrin And Metalloproteinase-containing family of proteins; ADAMTS, A Disintegrin And Metalloproteinase with ThromboSpondin motif-containing family of proteins; IGD, interglobular domain of aggrecan; MMP, matrix metalloproteinase
for the other. The answers to these questions led us to test the hypothesis that sequences surrounding
the MMP cleavage site may be important for aggrecanase activity.
Experimental Procedures

Materials

A baculovirus expression system was from Clontech USA. SF 9000 II serum free medium was from GIBCO-BRL USA. Restriction endonucleases, human interleukin-1α (1457-748) and chemiluminescence blotting kit were from Boehringer Mannheim, Germany. The ECL-plus enhanced chemiluminescence kit was from Amersham, UK. AmpliCycle Sequencing kit, and Taq DNA polymerase were from Perkin Elmer USA. Oligonucleotides were synthesised by Bresatec, Australia. The BioSil SEC-400 (600 x 21.5 mm) column was from Biorad, USA. Geneclean was from BIO 101 Inc., CA, USA. Hyaluronan-coupled-Sepharose (HA-Sepharose) was kindly provided by Professor T. Hardingham, University of Manchester, UK. The following reagents were generously provided by Professor G. Murphy, University of East Anglia, Norwich, UK: recombinant TIMP-1 (37), recombinant human proMMP-1 (38), recombinant human proMMP-3 (39), recombinant human proMMP-7 (40), proMMP-2 and proMMP-9 purified from human gingival fibroblast conditioned media (41). Recombinant human proMMP-13 (42) and recombinant human proMMP-8 were gifts from Dr. V. Knäuper and Professor G. Murphy, University of East Anglia, Norwich, UK. Recombinant ΔMT1-MMP (MMP-14) (43) was a gift from Prof. M Seiki and Prof. Y. Okada. The snake venom haemorrhagic toxin Ht-d (atrolysin C) was purified from rattlesnake venom (44) and kindly provided by Prof. J. Fox, University of Virginia, USA. Monoclonal AF-28 specific for the N-terminal sequence F<sub>342</sub>FGVG (45), polyclonal anti-ITEGE<sub>373</sub> (46) and polyclonal anti-DIPEN<sub>341</sub> (46) rabbit sera, were as described. Monoclonal antibody BC-3 (47) specific for the N-terminal sequence A<sub>374</sub>RGSV was a gift from Prof. B. Caterson and Dr. C. Hughes, University of Wales, Cardiff, UK. All other reagents were of analytical grade.

Site-directed Mutagenesis of the MMP and aggrecanase cleavage sites in the aggrecan IG D

Mutations in the human G1-G2 construct (46) were produced using splicing by overlap extension (SOE) PCR (48). The primer sets and their relative positions are shown in Table 1. Primers Aggr S12 and Aggr S13 introduced a twelve base deletion of GAAAACTTCTTT, resulting in deletion of four amino acids ENFF<sub>343</sub>. Primers Aggr S8 and Aggr S9 were used to replace CCAGAAAAC with GGATCAGCC, changing amino acids PEN<sub>341</sub> to GSA<sub>341</sub>. Primers Aggr S14 and Aggr S15 were used to replace TTCTTTGGA with GGCACTAGA, changing amino acids F<sub>342</sub>FG to G<sub>342</sub>TR. Primers Aggr S10 and Aggr S11 were used to replace GCCCGAGGC with AACGTATAC, changing amino acids A<sub>374</sub>RG to N<sub>374</sub>VY. Each mutation resulted in the introduction or removal of restriction enzyme sites to facilitate screening.

The polymerase chain reactions were done using a Perkin Elmer thermal cycler model 2400 or 9600, over 30 cycles. Cycle one was performed at 94° C for 120s, annealing (Table 1) for 90s and 72°C for 90s, followed by 30 cycles of 94°C for 30s, annealing (Table 1) for 45s and 72°C for 30s. The reactions were terminated at 72°C for 7min. Two independent PCR products were first produced using Aggr 7 (sense) with primer A (antisense), and primer B (sense) with Aggr 6 (antisense) of the primer sets in the first round of PCR using 10ng pBsktG1-G2<sup>46</sup> as a template. The amplified products were purified by agarose electrophoresis, recovered using Genecele and subjected to a second round of overlapping
PCR with primers Aggr 6 and Aggr 7. The PCR fragments were purified and digested with Spel and BsmI restriction enzymes to release a 375-bp fragment which was then subcloned into pBsktG1-G2, replacing the normal 375-bp cassette. The pBsktG1-G2 mutants were then digested with EcoRI and XbaI and the G1-G2 mutant construct subcloned into the pBacPAK8 transfer vector. Prior to production of recombinant virus, the mutant constructs were screened by restriction enzyme digestion and sequenced using AmpliTag Cycle sequencing. Wildtype and mutant rG1-G2 were expressed and purified as described (46).

Proteinase digestions of wildtype and mutant rG1-G2
Matrix metalloproteinase, aggrecanase and atrolysin C digestions were done at 37°C in buffer containing 10mM calcium chloride, 100mM sodium chloride, 50mM Tris-HCl, pH 7.5. Digests were stopped either by boiling, or adding EDTA and 1,10 phenanthroline to final concentrations of 10mM and 2mM, respectively. Denatured samples were analysed by Western blotting or silver stain after SDS-PAGE. In sequential digest experiments, 10µg substrates digested with 3µg/ml MMP-13 for 2 hours were incubated with a two-fold molar excess of TIMP-1 for 30 minutes on ice to inhibit further MMP-13 action, then incubated overnight with 3µl aggrecanase. Similarly, substrates digested overnight with 3µl aggrecanase were subsequently digested for 2 hours with 3µg/ml MMP-13.

Preparation of bovine nasal aggrecanase
Aggrecanase was purified from bovine nasal cartilage in a similar fashion to that previously reported (4). In brief, nasal cartilage was dissected from bovine noses obtained within 4 hours after slaughter. The tissue was cut into 1.2mm cubed pieces and cultured for 2 days in DMEM containing 5% FCS, followed by 1 day in DMEM containing 2.5% FCS and finally 1 day in DMEM without FCS. After the one day in serum-free medium, IL-1α was added at a concentration of 0.15nM. The conditioned medium was collected 48 hours later and aggrecanase was purified by ion exchange, gel filtration and wheatgerm agglutinin chromatography. Several silver stained bands were present on SDS gels however the preparation was “proteolytically pure” since it contained no MMP activity and other classes of proteinase inhibitors failed to reduce the amount of ITEGE₃₇₃ products formed. The aggrecanase preparation was not inhibitable by TIMP-1 when assessed using bovine aggrecan as substrate and anti-ITEGE₃₇₃ neoepitope as the readout. TIMP-1 at 2.2µM failed to inhibit the aggrecanase activity but gave an IC₅₀ against MMP-1 and MMP-8 of 4nM and <2nM respectively, using a synthetic substrate assay. Since TIMP-1 has an IC₅₀ of 210nM against ADAMTS-4 (personal communication, Dr. Elizabeth Arner), it is unlikely that the aggrecanase preparation used in the present work is identical to ADAMTS-4 (3).

Inhibition of aggrecanase activity by synthetic peptides
The 7mer peptides IPENFFG and TEGEARG were from Charing Cross Hospital London, UK and the 10mer peptide FVDIPENFFG was from Auspep, Australia. IPENFFG and FVDIPENFFG were dissolved in distilled water at 25mg/ml and TEGEARG was dissolved in 75mM NaCO₃, pH8.0 at the same concentration. The peptides were present at a 3,000 fold molar excess over rG1-G2 substrate in
aggrecanase digests. Addition of 75mM NaC0_3 buffer alone to aggrecanase digests did not alter the pH, nor did it affect the generation of ITEGE_373 epitope.
Results
Mutations at the DIPEN<sub>341</sub> $\downarrow$ FFGVG and ITEGE<sub>373</sub> $\downarrow$ ARGSV cleavage sites in the aggrecan IGD were made (Figure 1) and the mutant substrates tested for their susceptibility to digestion by MMPs, aggrecanase and atrolysin C.

**MMP-13 digestion of wildtype and mutant rG1-G2**
Wild-type and mutant G1-G2 substrates were digested with MMP-13 since this MMP is abundantly expressed in arthritic cartilage. Silver staining (Figure 2a,d,g,j,m) was used to monitor (a) the extent of digestion and the approximate ratio of undigested:digested material, and (b) uniform loading of samples on gels. Silver staining was not useful for specifically identifying G1 and G2 domains as the fragments often migrated together. Western blotting with neoepitope antibodies was used to determine whether cleavage was occurring at the MMP site. Mutation of the sequence DIPEN<sub>341</sub> to DIGSA<sub>341</sub> retarded, but did not block, cleavage at the MMP site, since a higher concentration of MMP-13 (10$\mu$g/ml) was required to produce the maximum amount of F<sub>342</sub>FGVG epitope in digests of the DIGSA<sub>341</sub> mutant (Figure 2e) compared with the wildtype (Figure 2b). However mutation of F<sub>342</sub>FGVG to G<sub>342</sub>TRVG completely abolished cleavage at the MMP site since no DIPEN<sub>341</sub> epitope was detected in digests of the G<sub>342</sub>TRVG mutant (Figure 2i). This result is consistent with previous reports that most substitutions at the P<sup>1</sup> position are detrimental for collagenase activity (49). Silver staining showed that the G<sub>342</sub>TRVG mutant was cleaved by MMP-13 elsewhere in the IGD, most likely at the minor sites P<sub>384</sub>↓V<sub>385</sub> and D<sub>441</sub>↓L<sub>442</sub> as shown for native G1-G2 (11). Generation in the N<sub>374</sub>VYSV mutant of DIPEN<sub>341</sub> and F<sub>342</sub>FGVG neoepitopes by MMP-13 (Figure 2m,n,o) was similar to the wildtype (Figure 2a,b,c), indicating that the N<sub>374</sub>VYSV mutation at the aggrecanase site had no affect on MMP-13 activity against rG1-G2. MMP-13 at 100$\mu$g/ml appeared to “over-digest” the substrate, leading to loss of F<sub>342</sub>FGVG (Figure 2b,e,n) and DIPEN<sub>341</sub> (Figure 2c,o) neoepitopes as well as G1 domain epitopes detected with polyclonal antisera (data not shown). This is consistent with our previous observation that rG1-G2 is more sensitive to proteolysis than native glycosylated pig G1-G2 (46).

**MMP-8 digestion of wildtype and mutant rG1-G2**
MMP-8 cleaves bovine aggrecan (50) and native pig G1-G2 (51) at both the MMP and aggrecanase sites. MMP-8 cleaves its substrate in a sequential manner, at the N<sub>341</sub>↓F bond initially, and the E<sub>373</sub>↓A bond subsequently. Wildtype rG1-G2 was also cleaved in a sequential manner by MMP-8. F<sub>342</sub>FGVG epitope was maximal at low concentrations of enzyme but decreased at higher concentrations (Figure 3b) and the decrease in epitope was concomitant with an increase in A<sub>374</sub>RGSV epitope (Figure 3d). DIPEN<sub>341</sub> epitope on the other hand was unchanged (Figure 3c). As with MMP-13, the DIGSA<sub>341</sub> mutant was less susceptible to MMP-8 cleavage at the MMP site. Higher concentrations of MMP-8 were required to achieve maximum F<sub>342</sub>FGVG epitope in the DIGSA<sub>341</sub> digests and epitope levels were not noticeably decreased at the highest concentration of enzyme (200$\mu$g/ml)(Figure 3f), even though a small amount of A<sub>374</sub>RGSV epitope was detected (Figure 3h). MMP-8 digestion of the G<sub>342</sub>TRVG mutant showed that hydrolysis of the N<sub>341</sub>↓F bond was not an essential prerequisite for cleavage at
E$_{373}$\textdownarrow A. A$_{374}$RGSV epitope was detected in digested G$_{342}$TRVG mutant at enzyme concentrations of 30µg/ml and higher (Figure 3l), in the absence of MMP site cleavage (Figure 3k). The N$_{374}$VYSV mutation did not interfere with MMP-8 cleavage at DIPEN$_{341}$\textdownarrow FFGVG, but did block cleavage at ITEGE$_{373}$\textdownarrow ARGSV as there was no loss of F$_{342}$FGVG epitope at high concentrations of enzyme (Figure 3r).

To determine whether the G$_{342}$TRVG mutant resisted cleavage by other MMPs, wild-type and mutant rG1-G2 were next digested with a single concentration of MMP-1, -2, -3, -7, -9 and –14 (Figure 4). Silver staining showed that MMP-1, MMP-7 and MMP-14 cleaved the GTR mutant (Figure 4g), however the lack of DIPEN$_{341}$ reactivity in the digests showed that none of the MMPs cleaved at the mutated MMP site (Figure 4i). Some F$_{342}$FGVG epitope was present in DIGSA$_{341}$ mutants digested with MMP-2, MMP-3 and MMP-14 (Figure 4e). The data show that mutation of DIPEN$_{342}$FGVG to DIPENG$_{342}$TRVG blocked cleavage at the major MMP site by three collagenases, two gelatinases, stromelysin-1, matrilysin and MT1-MMP.

Aggrecanase digestion of wildtype and mutant rG1-G2

Wildtype and mutant rG1-G2 were incubated with increasing amounts of bovine aggrecanase and the products detected by Western blotting with anti-ITEGE$_{373}$ and anti-A$_{374}$RGSV antibodies. All the rG1-G2 substrates except the N$_{374}$VYSV mutant were efficiently cleaved by aggrecanase at the ITEGE$_{373}$\textdownarrow ARGSV site (Figure 5). Silver staining showed that no N$_{374}$VYSV degradation products were produced by aggrecanase (Figure 5m) suggesting that, unlike the MMPs, aggrecanase had specificity for only one site in the IGD.

Atrolysin C digestion of wildtype and mutant rG1-G2

Native glycosylated bovine aggrecan (52) and pig G1-G2 (46) are cleaved by atrolysin C at both the DIPEN$_{341}$\textdownarrow FFGVG and ITEGE$_{373}$\textdownarrow ARGSV bonds, in an independent rather than sequential manner. However we have recently found that wildtype rG1-G2, which is largely unglycosylated, is cleaved by atrolysin C only at ITEGE$_{373}$\textdownarrow ARGSV and not DIPEN$_{341}$\textdownarrow FFGVG (46). In the present experiments, all the rG1-G2 substrates except the N$_{374}$VYSV mutant (Figure 6n), were cleaved by atrolysin C at ITEGE$_{373}$\textdownarrow ARGSV, as detected by anti-ITEGE$_{373}$ immunoreactivity (Figure 6b,e,h,k). No DIPEN$_{341}$ epitope was detected in any of the digests (data not shown), suggesting that atrolysin C was not able to cleave at DIPEN$_{341}$\textdownarrow FFGVG in the wildtype, G$_{342}$TRVG or N$_{374}$VYSV substrates. Surprisingly, F$_{342}$FGVG immunoreactivity was detected in digested DIGSA$_{341}$ mutant (Figure 6f). These results suggest that the conformational shape of the substrate surrounding the sequence DIPEN$_{341}$ may dictate atrolysin C specificity for cleavage in the IGD.

Relationship between MMP and aggrecanase activities in the IGD

In order to further examine the relationship between proteolysis at the N$_{342}$\textdownarrow F and E$_{373}$\textdownarrow A bonds, we designed experiments to test whether aggrecanase-derived G1 fragments (with ITEGE$_{373}$ C-terminus)
were viable substrates for MMPs (Figure 7) and conversely, whether MMP-derived G2 fragments (with $F_{342}^\text{FGVG}$ N-terminus) were viable substrates for aggrecanase (Figure 8). We predicted that if aggrecanase-G1 fragments were digested by MMPs (Figure 7c), we would create DIPEN$_{341}$ epitope, lose ITEGE$_{373}$ epitope (present on the 3 kDa fragment) and observe no change in A$_{374}^\text{RGSV}$ epitope (Figure 7d,h). The Western blots revealed that DIPEN$_{341}$ epitope was indeed increased (Figure 7e, lanes 3 & 4) while ITEGE$_{373}$ epitope was completely destroyed following digestion of aggrecanase-G1 with MMP-13 (Figure 7f, lanes 3 & 4). These results therefore confirm that the aggrecanase-derived G1 domain is a viable substrate for MMPs.

In contrast we found that the MMP-G2 domain was not digested by aggrecanase. We predicted that if MMP-G2 fragments were digested by aggrecanase (Figure 8c), we would create A$_{374}^\text{RGSV}$ epitope, lose $F_{342}^\text{FGVG}$ epitope (present on the 3kDa fragment) and observe no change in DIPEN$_{341}$ epitope (Figure 8d,e). However Western blot analysis showed that $F_{342}^\text{FGVG}$ epitope was not destroyed (Figure 8f, lanes 2 & 4) and there was no gain in A$_{374}^\text{RGSV}$ epitope (Figure 8i, lane 4). The small amount of anti-A$_{374}^\text{RGSV}$ epitope present in Figure 8i lane 4 is most likely derived from cleavage of intact substrate, rather than MMP-G2 domain, as some undigested substrate survives digestion with MMP-13 for 2 hours at 3 $\mu$g/ml (Figure 2a). Furthermore, pretreatment of rG1-G2 with MMP-13 (Figure 8i, lane 4) markedly reduced the yield of A$_{374}^\text{RGSV}$ epitope by aggrecanase (compare Figure 8i lane 4 with Figure 8i lane 3). Aggrecanase was active in the presence of TIMP-inhibited MMP-13, since G$_{342}^\text{TRVG}$ mutants digested under the same conditions gave A$_{374}^\text{RGSV}$ epitope (Figure 8j, lane 4). The results show that the MMP-G2 domain is not a viable substrate for aggrecanase.

**Sequences in the MMP-G1 domain are required for aggrecanase activity**

A previous study in our laboratory showed that a 7-mer peptide $\text{IPENFFG}$, a substrate analogue for the MMP cleavage site, was able to inhibit the release of A$_{374}^\text{RGSV}$ fragments from cartilage cultured with and without interleukin-1 (51). The results of this experiment suggested that the $\text{IPENFFG}$ peptide was able to inhibit aggrecanase, and now, in conjunction with the results presented in Figure 8, raises the possibility that sequences present at the C-terminus of the MMP-G1 domain may be necessary for aggrecanase activity. To test this hypothesis, we used several synthetic peptides as competitive substrates in aggrecanase digests of rG1-G2 (Figure 9). In these experiments the $\text{IPENFFG}$ 7-mer peptide was unable to block aggrecanase cleavage at E$_{373}^\downarrow$ A (Figure 9b lane 3, c) however cleavage was inhibited by 50% in the presence of a 10-mer peptide with sequence $\text{FVDIPENFFG}$ (Figure 9b lane 4, c). A 7-mer peptide $\text{TEGEARG}$, a substrate analogue for the aggrecanase site, completely blocked aggrecanase cleavage.
Discussion

Our finding that MMP-derived G2 fragments are resistant to aggrecanase cleavage in the IGD is novel and provides an explanation for our earlier observation (36,34) that IL-1α-induced loss of aggrecan from pig articular cartilage by MMPs and aggrecanase appeared to be mutually exclusive. The present findings are not limited to digestion of rG1-G2, since native glycosylated F_{342}FGVG fragments (see companion paper, Figure 7) and native deglycosylated MMP-1 digested aggrecan (29) also resist digestion by aggrecanase. To revisit the models outlined in the introduction; model 1, in which aggrecan is cleaved initially by aggrecanase and the G1 fragments remaining in the tissue are subsequently cleaved by MMPs, appears viable. However the subsequent MMP cleavage in this model represents processing of the G1 domain and has no effect on further loss of aggrecan from the matrix. Model 2, in which aggrecan is cleaved initially by MMPs and the released fragment containing the F_{342}FGVG neoepitope is subsequently cleaved by aggrecanase, seems unlikely. Our finding that recombinant F_{342}FGVG fragments and native pig F_{342}FGVG fragments (see companion paper) resist aggrecanase cleavage in the IGD thus extends the independent model, model 3. The results show that, in terms of aggrecan loss from tissue (as opposed to ITEGE_{373} G1-domain processing), MMP and aggrecanase activities appear to be mutually exclusive in IL-1α-stimulated aggrecan release from pig cartilage and aggrecanolysis of rG1-G2 in vitro. The results are also consistent with our finding that small F_{342}FGVG fragments detected in synovial fluids of osteoarthritis and inflammatory arthritis patients do not contain an ITEGE_{373} C-terminus (19).

In principle, these results have implications for therapeutic strategies designed to limit aggrecan loss, since it appears that inhibition of both activities may be required. Our results with the mutant G1-G2 substrates also suggest that abrogation of one activity has no consequence for the other activity. A spectrum of sizes of degraded aggrecan fragments is present in synovial fluids of arthritis patients. The large, high buoyant density fragments that can be recovered from synovial fluids fractionated on caesium chloride density gradients are predominantly aggrecanase-derived and do not contain any fragments with F_{342}FGVG N-termini (16,17). Low buoyant density fragments fractionated on caesium chloride density gradients do contain small F_{342}FGVG fragments (19), and since these fragments do not carry the ITEGE_{373} C-terminus, it is possible they are the products of more extensive MMP processing. Studies of aggrecanolysis in chondrocyte and cartilage explant cultures show that aggrecanase is the predominant activity in vitro (35,29)(see companion paper), however the detection (45) and quantitation (19) of F_{342}FGVG fragments in human synovial fluids, or released from IL-1α-treated human OA cartilage (29), suggests that MMPs may play a greater role in aggrecanolysis in human disease, than in in vitro animal models. The relative involvement of MMPs and aggrecanase in arthritis remains unclear.

The processing of ITEGE_{373} G1 to DIPEN_{341} G1 is likely to occur in vivo. Immunolocalisation studies in mice with experimentally induced arthritis have shown that ITEGE_{373} neoepitopes were less prominent in areas showing advanced cartilage damage, compared with DIPEN_{341} epitope, and that when intense DIPEN_{341} staining appeared, ITEGE_{373} epitope disappeared (26). These results suggest that either ITEGE_{373} G1 is cleared quickly from the tissue, or that the G1 fragment is rapidly cleaved by MMPs or
other proteinases, destroying the epitope. Under conditions where the tissue pH is acidic, the increased DIPEN\textsubscript{341} epitope and concomitant loss of ITEGE\textsubscript{373} epitope could arise from the endo and exopeptidase activity of cathepsin B (53).

The peptide experiment (Figure 9), together with our previous observation that the 7-mer peptide IPENFFG was able to inhibit the release of A\textsubscript{374}RGSV fragments from cartilage in culture (51), suggests that sequences present at the C-terminus of the FVDIPEN\textsubscript{341} G1 domain maybe important for aggrecanase activity. One possibility is that these sequences provide a docking site for the enzyme. If the P\textsubscript{339}, E\textsubscript{340} or N\textsubscript{341} residues were critical for aggrecanase docking or activity we would expect that cleavage at the ITEGE\textsubscript{373} \textarrow{↓} ARGSV site may have been reduced in the DIGSA\textsubscript{341} and deletion mutants. This was not the case, suggesting therefore that sequences other than P-E-N are involved. This is consistent with our observation that a longer peptide with sequence FVDIPENFFG was effective in inhibiting aggrecanase activity by 50\%, while the shorter IPENFFG peptide had no effect in the present style of experiment. An alternative explanation is that the longer sequence allows the formation of a peptide with secondary or tertiary structure and that it is the conformational shape rather than the peptide sequence that is important. This could be addressed by determining whether a peptidic hydroxamate inhibitor based on the P-E-N residues could inhibit aggrecanase.

Three MMP cleavage site mutants were made and these partially or totally blocked cleavage at the DIPEN\textsubscript{341} \textarrow{↓} FFGVG site, however none of the mutations conferred complete protection from degradation by MMP-8 or –13, as seen in the fragmentation pattern by silver stain. The enzymes were clearly able to cleave elsewhere in the IGD, possibly at the minor sites (10,11). The G\textsubscript{342} TRVG mutant resisted cleavage by stromelysin-1, two gelatinases, three collagenases, matrilysin and MT1-MMP at the mutated MMP site. The DIGSA\textsubscript{341} mutant was partially resistant to some of these MMPs. In the future it will be interesting to determine whether different glycosylation affects MMP or aggrecanase specificity for cleavage in the IGD.

In contrast to the MMP cleavage site mutants, the N\textsubscript{374}VYSV mutant was not cleaved at all by aggrecanase, showing that aggrecanase has specificity for only a single site in the IGD. Our ongoing studies generating an N\textsubscript{374}VYSV knock-in mouse will enable us to further explore the role of aggrecanase in normal growth and development and also in the initiation and progression of arthritis.
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We wish to express our gratitude to Prof. G. Murphy, Dr. V. Knäuper, Prof. J. Fox, Prof. M. Seiki, Prof. Y. Okada, Prof. T. Hardingham, Prof. B. Caterson and Dr. C. Hughes for the reagents they provided. We acknowledge the National Health and Medical Research Council (Australia), the Arthritis Foundation of Australia and the Royal Children’s Hospital Research Institute for financial support.
References


Figure Legends

**Figure 1** Sequence and position of the amino acid substitutions at the MMP and aggrecanase cleavage sites in the IGD

**Figure 2** MMP-13 digestion of wildtype and mutant rG1-G2
Recombinant G1-G2 (5µg) was digested for 2 hours at 37°C with the concentrations of MMP-13 shown, in a total volume of 20µl. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analysed by silver staining (a,d,g,j,m) or Western blotting with monoclonal anti-F$_{342}$FGVG (b,e,h,k,n) or anti-DIPEN$_{341}$ antisera (c,f,i,l,o). The slashed boxes indicate that the epitope sequence was absent in a particular mutant.

**Figure 3** MMP-8 digestion of wildtype and mutant rG1-G2
Recombinant G1-G2 (10µg) was digested for 2 hours at 37°C with the concentrations of MMP-8 shown, in a total volume of 20µl. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analysed by silver staining (a,e,i,m,q) or Western blotting with monoclonal anti-F$_{342}$FGVG (b,f,j,n,r), anti-DIPEN$_{341}$ antisera (c,g,k,o,s) or monoclonal anti-A$_{374}$RGSV (d,h,l,p,t).

**Figure 4** MMP digestion of wildtype and mutant rG1-G2
Recombinant G1-G2 (2.5µg) was digested for 2 hours at 37°C with 125µg/ml MMP-1, 100µg/ml MMP-2, 125µg/ml MMP-3, 86.7µg/ml MMP-7, 130.5µg/ml MMP-9 or 75µg/ml MMP-14 in a total volume of 10µl. Aliquots of digested and undigested rG1-G2 were electrophoresed on 5% SDS gels and analysed by silver staining (a,d,g,j) or Western blotting with monoclonal anti-F$_{342}$FGVG (b,e,h,k) or anti-DIPEN$_{341}$ antisera (c,f,i,l).

**Figure 5** Aggrecanase digestion of wildtype and mutant rG1-G2
Recombinant G1-G2 (5µg) was digested for 21 hours at 37°C with the volumes of purified aggrecanase shown, in a total volume of 20µl. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analysed by silver staining (a,d,g,j,m) or Western blotting with anti-ITEGE$_{373}$ (b,e,h,k,n) or monoclonal anti-A$_{374}$RGSV (c,f,i,l,o).

**Figure 6** Atrolysin C digestion of wild-type and mutant rG1-G2
Recombinant G1-G2 (5µg) was overnight at 37°C with concentrations of atrolysin C as shown, in a total volume of 20µl. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analysed by silver staining (a,d,g,j,m) or Western blotting with anti-ITEGE$_{373}$ (b,e,h,k,n) or monoclonal anti-F$_{342}$FGVG (c,f,i,l,o).
**Figure 7  Aggrecanase followed by MMP-13 sequential digest**

(a-d) Schematic diagram showing the predicted fragments and neoepitope sequences generated by MMP digestion of the aggrecanase-G1 domain. (e-g) Wild-type rG1-G2 was digested with 3µg/ml MMP-13 for 2 hours (lane 2), 3µl aggrecanase for 21 hours (lane 3) or 3µl aggrecanase for 21 hours followed by 3µg/ml MMP-13 for 2 hours (lane 4). Aliquots were electrophoresed on SDS gels and analysed by Western blotting with anti-DIPEN$_{341}$ (e) anti-ITEGE$_{373}$ (f) or monoclonal anti-A$_{374}$RGSV (g). (h) The expected and observed outcome for the products obtained in panel (d) and lane 4 relative to panel (b) and lane 3 is shown.

**Figure 8  MMP-13 followed by aggrecanase sequential digest**

(a-d) Schematic diagram showing the predicted fragments and neoepitope sequences generated by aggrecanase digestion of the MMP-G2 domain. (f-i) Wild-type rG1-G2 was digested with 3µg/ml MMP-13 for 2 hours (lane 2), 3µl aggrecanase for 21 hours (lane 3) or 3µg/ml MMP-13 for 2 hours followed by 3µl aggrecanase for 21 hours (lane 4). (e) The expected and observed outcome for the products obtained in panel (d) and lane 4 relative to panel (b) and lane 2 is shown. Aliquots were electrophoresed on SDS gels and analysed by Western blotting with monoclonal anti-F$_{342}$FGVG (f), anti-DIPEN$_{341}$ (g), anti-ITEGE$_{373}$ (h) or monoclonal anti-A$_{374}$RGSV (i). GTRGV mutant G1-G2 was digested with 3µg/ml MMP-13 for 2 hours (lane 6), 3µl aggrecanase for 21 hours (lane 7) or 3µg/ml MMP-13 for 2 hours followed by 3µl aggrecanase for 21 hours (lane 8). Aliquots were electrophoresed on SDS gels and analysed by Western blotting with monoclonal anti-A$_{374}$RGSV (j).

**Figure 9  Competitive inhibition of aggrecanase by MMP-site peptide analogues**

Wild-type rG1-G2 was digested with aggrecanase (3µl) for 17 hours at 37°C with or without synthetic peptides, in a 3,000 fold molar excess over rG1-G2, as shown. Digested and undigested samples were electrophoresed on SDS gels and analysed by silver staining (a) or Western blotting with anti-ITEGE$_{373}$ antisera (b). The inhibition of aggrecanase activity was determined by densitometric analysis of the ITEGE$_{373}$ fragments.
<table>
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<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Altered amino acid sequence</th>
<th>Primer Location</th>
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**Table 1  Primers for SOE PCR**

Sequences in bold are the annealing sequences in the primary PCR. Sequences in italics are the overlapping sequences in the secondary PCR. The annealing temperatures for the primary reactions are shown and all overlap secondary PCR reactions were done at 60°C. The primer locations are based on numbering from the published sequence (54).
Figure 1
Figure 2

[Image of gel electrophoresis results for Wildtype, GSA mutant, GTR mutant, ΔMMP mutant, and NVY mutant with corresponding silver stain and antibody labeling for FFGVG and DIPEN]
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Mutations in the interglobular domain of aggrecan alters matrix metalloproteinase and aggrecanase cleavage patterns: Evidence that matrix metalloproteinase cleavage interferes with aggrecanase activity
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