Mutations in the Interglobular Domain of Aggrecan Alter Matrix Metalloproteinase and Aggrecanase Cleavage Patterns

EVIDENCE THAT MATRIX METALLOPROTEINASE CLEAVAGE INTERFERES WITH AGGRECANASE ACTIVITY*

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We have expressed G1-G2 mutants with amino acid changes at the DIPEN341^342FFGVG and ITEGE73^74.

ARGSV cleavage sites, in order to investigate the relationship between matrix metalloproteinase (MMP) and aggrecanase activities in the interglobular domain (IGD) of aggrecan. The mutation DIPEN341 to DIGSA341 partially blocked cleavage by MMP-1 and MMP-8 at the MMP site, while the mutation 342FFGVG to 342GTRVG 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 ENFF343, 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 374ARGSV to 374NVYSV 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-3 to cleave at the Asn374^Phe bond. Susceptibility to atrolysin C cleavage at the MMP site was conferred in the DIGSA341 mutant but absent in the wild-type, 342GTRVG, 374NVYSV, 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 ITEGE73 C termini were viable substrates for MMPs; however, MMP-derived G2 fragments were resistant to cleavage by aggrecanase. A 10-mer peptide FVDPENFFFG, which is a substrate analogue for the MMP cleavage site, inhibited aggrecanase cleavage at the Glu733^Ala 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 DIPEN341 G1 domain may be important for regulating aggrecanase cleavage.

The large proteoglycan aggrecan is removed from cartilage

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The abbreviations used are: MMP, matrix metalloproteinase; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs-containing family of proteins; IGD, interglobular domain of aggrecan; PCR, polymerase chain reaction; IL, interleukin; rG1-G2, recombinant G1-G2.

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MMP and Aggrecanase Cleavage Site Mutants in rG1-G2

Sequences in boldface 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 PCRs were done at 60 °C. The primer locations are based on numbering from the published sequence (54).

### Table I

| Primer | Primer sequence | Altered amino acid sequence | Primer location | Annealing temperature in primary PCR | Unique restriction site lost or gained |
|--------|-----------------|-----------------------------|-----------------|-------------------------------------|--------------------------------------|
| Aggr S8-A | 5’-GGATCAGCCCTCTTGGGA-3’ | Forward primer used to create the DIPEN-DIGSA mutant | 1072–1095 | 60 | −Fok I |
| Aggr S9-B | 5’-GAGATGGCATTCCGATGCCCACAAA-3’ | Reverse primer for above | 1083–1060 | 50 |
| Aggr S10-A | 5’-GGTGAAACGTATACCGGATGATCCTTACC-3’ | Forward primer used to create the ARGVV-UVYSV mutant | 1171–1200 | 65 | +Acc I |
| Aggr S11-B | 5’-GGTGTATCTTCCCTCAGCCTCAGTGAT-3’ | Reverse primer for above | 1188–1162 | 60 |
| Aggr S12-A | 5’-TTTGATGACACCTCCAGAGTTGGGAGGATGAG-3’ | Forward primer used to create the ENFF deletion mutant | 1062–1098 | 60 | +Eco RI |
| Aggr S13-B | 5’-CTTACCCCCACCTCCTGGGATGCCACAA-3’ | Reverse primer for above | 1106–1062 | 60 |
| Aggr S14-A | 5’-CCAGAAAACGCGACTAGAGTGCTGGG-3’ | Forward primer used to create the FFGVG-VTRVG mutant | 1074–1106 | 60 | +Mae II |
| Aggr S15-B | 5’-ACCCCCCCACTCTGATCCCCTTCTGG-3’ | Reverse primer for above | 1100–1071 | 60 |
| Aggr 6-A | 5’-CAGAAAGCAAGCAGCCATGAGTTGGG-3’ | Forward primer | 798–818 |
| Aggr 7-B | 5’-AAGCCGGCTCCCAAGGCCA-3’ | Reverse primer | 1421–1399 | 60 |

EXPERIMENTAL PROCEDURES

Materials—A baculovirus expression system was from CLONTECH. SF 9000 II serum-free medium was from Life Technologies, Inc. Restriction endonucleases, human interleukin-1α (1-457-748), and chemiluminescence blotting kit were from Roche Molecular Biochemicals. The ECL-plus enhanced chemiluminescence kit was from Amersham Pharmacia Biotech. AmpliCycle Sequencing kit and Taq DNA polymerase were from PerkinElmer Life Sciences. Oligonucleotides were synthesized by Bresatec, Australia. The BioSil SEC-400 (600 × 21.5 mm) column was from Bio-Rad. Genelean was from Bio 101 Inc. Hyaluronic acid was kindly provided by Professor T. Hardingham (University of Manchester, United Kingdom). 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 medium (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). Recombinant AMT1-MMP (MMP-14) (43) was a gift from Prof. M. Seiki and Prof. Y. Okada. The snake venom hemorrhagic toxin Hε-d (atrolysin C) was purified from rattlesnake venom (44) and kindly provided by Prof. J. Fox (University of Virginia, Charlottesville, VA). Monoclonal AF-28 specific for the N-terminal sequence 342FFG (44) and polyclonal anti-ITEGE373 (46), and polyclonal anti-DIPEN341 (46) rabbit sera were as described. Monoclonal antibody BC-3 (47) specific for the N-terminal sequence 374ARGSV 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 IGDMutations in the human G1-G2 construct (46) were produced using splicing by overlap extension PCR (48). The primer sets and their relative positions are shown in Table 1. Primers Aggr S12 and Aggr S13 introduced a 12-base deletion of GAAAACCTCTCTTCTGG, resulting in deletion of four amino acids, ENFF343. Primers Aggr S10 and Aggr S15 were used to replace TTCTTT, resulting in deletion of five amino acids, ENFF341. Primers Aggr S8 and Aggr S9 were used to replace CCAGAAAACCGGACTAGAGTGCTGGG with GCGACTAGCC, changing amino acids PEN341 to GSA341. Primers Aggr S14 and Aggr S15 were used to replace TTCTTTGGGA with GGCACTAGAGTGCTGGG, changing amino acids FFGVG to VTRVG. 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 10 ng of pBsktG1-G2 (46) as a template. The amplified products were purified by agarose electrophoresis, recovered using Genelean, and subjected to a second round of overlapping PCR with primers Aggr 6 and Aggr 7. The PCR fragments were purified and digested with SphI and BsmI restriction enzymes to release a 375-bp fragment, which was then subcloned into pBluescript SK+ (Stratagene) for sequencing.

cartilage in vitro. Since the ITEGE373 G1 domain and 342FFGVG fragments cannot be derived from the one aggrecan molecule, we concluded that in pig cartilage stimulated with IL-1a, 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 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.
into pBsktG1-G2, replacing the normal 375-bp cassette. The pBsktG1-G2 mutants were then digested with EcoRI and XhoI, and the G1-G2 mutant construct was subcloned into the pBacPARK8 transfer vector. Prior to production of recombinant virus, the mutant constructs were screened by restriction enzyme digestion and sequenced using AmpliTaq Cycle sequencing. Wild-type and mutant rG1-G2 were expressed and purified as described (46).

Proteasease Digestions of Wild-type and Mutant rG1-G2—Matrix metalloproteinase, aggrecanase, and atrolysin C digestions were conducted at 37 °C in buffer containing 10 mM calcium chloride, 100 mM sodium chloride, 50 mM Tris-HCl, pH 7.5. Digests were stopped either by boiling or by adding EDTA and 1,10-phenanthroline to final concentrations of 10 and 2 mM, respectively. Denatured samples were analyzed by Western blotting or silver stain after SDS-polyacrylamide gel electrophoresis. In sequential digest experiments, 10 μg of substrates digested with 3 μg/ml MMP-13 for 2 h were incubated with a 2-fold molar excess of TIMP-1 for 30 min on ice to inhibit further MMP-13 action and then incubated overnight with 3 μl of aggrecanase. Similarly, substrates digested overnight with 3 μl of aggrecanase were subsequently digested for 2 h with 3 μg/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 h after slaughter. The tissue was cut into 1.2-mm cubed pieces and cultured for 2 days in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, followed by 1 day in Dulbecco’s modified Eagle’s medium containing 2.5% fetal calf serum and finally 1 day in Dulbecco’s modified Eagle’s medium without fetal calf serum. After the 1 day in serum-free medium, IL-1α was added at a concentration of 0.15 nM. The conditioned medium was collected 48 h later, and aggrecanase was purified by ion exchange, gel filtration, and wheat germ agglutinin chromatography. Several silver-stained bands were present on SDS gels; however, the preparation was “protoenylly 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 neopeptide as the read-out. TIMP-1 at 2 μg/ml failed to inhibit the aggrecanase activity but gave an IC50 against MMP-1 and MMP-8 of 4 nM and <2 nM, respectively, using a synthetic substrate assay. Since TIMP-1 has an IC50 of 210 nM against ADAMTS-4, it is unlikely that the aggrecanase preparation used in the present work is identical to ADAMTS-4 (S).

Inhibition of Aggrecanase Activity by Synthetic Peptides—The 7-mer peptides IPFNGF and TEGARG were from Charing Cross Hospital (London), and the 10-mer peptide FVDIPEFNG was from Auspep. IPFNGF and FVDIPEFNG were dissolved in distilled water at 25 mg/ml, and TEGARG was dissolved in 75 mM NaCO3, pH 8.0, at the same concentration. The peptides were present at a 3000-fold molar excess over rG1-G2 substrate in aggrecanase digests. The addition of 75 mM NaCO3 buffer alone to aggrecanase digests did not alter the pH, nor did it affect the generation of ITEGE373 epitope.

RESULTS

Mutations at the DIPEN341 FFGVG and ITEGE373 ARGVS epitope cleavage sites in the aggrecan IGD were made (Fig. 1), and the mutant substrates were tested for their susceptibility to digestion by MMPs, aggrecanase, and atrolysin C.

MMP-13 Digestion of Wild-type 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 (Fig. 2, a, d, g, j, and m) was used to monitor 1) the extent of digestion and the approximate ratio of undigested:digested material and 2) uniform loading of samples on gels. Silver staining was not useful for specifically identifying G1 and G2 domains, since the fragments often migrated together. Western blotting with neopeptide antibodies was used to determine whether cleavage was occurring at the MMP site. Mutation of the sequence DIPEN341 to DIGSA341 retarded, but did not block, cleavage at the MMP site, since a higher concentration of MMP-13 (10 μg/ml) was required to produce the maximum amount of 342FFGVG epitope in digests of the DIGSA341 mutant (Fig. 2c) compared with the wild type (Fig. 2b). However, mutation of 342FFGVG to 342GTRVG completely abolished cleavage at the MMP site, since no DIPEN341 epitope was detected in digests of the 342GTRVG mutant (Fig. 2i). This result is consistent with previous reports that most substitutions at the P1 position are detrimental for collagenase activity (49). Silver staining showed that the 342GTRVG mutant was cleaved by MMP-13 elsewhere in the IGD, most likely at the minor sites Pro384Val385 and Asp441Leu442, as shown for native G1-G2 (11). Generation in the 373NVYSV mutant of DIPEN341 and 342FFGVG neopeptides by MMP-13 (Fig. 2, m–o) was similar to the wild type (Fig. 2, a–c), indicating that the 373NVYSV mutation at the aggrecanase site had no effect on MMP-13 activity against rG1-G2. MMP-13 at 100 μg/ml appeared to “overdigest” the substrate, leading to loss of 342FFGVG (Fig. 2, b, e, and n) and DIPEN341 (Fig. 2, c and o) neopeptides 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 Wild-type 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 Asn341[Pro] bond initially, and the Glu373[Ala] bond subsequently. Wild-type rG1-G2 was also cleaved in a sequential manner by MMP-8. 342FFGVG epitope was maximal at low concentrations of enzyme but decreased at higher concentrations (Fig. 3b), and the decrease in epitope was concomitant with an increase in 374ARGSV epitope (Fig. 3d). DIPEN341 epitope on the other hand was unchanged (Fig. 3c). As with MMP-13, the DIGSA341 mutant was less susceptible to MMP-8 cleavage at the MMP site. Higher concentrations of MMP-8 were required to achieve maximum 342FFGVG epitope in the DIGSA341 digests, and epitope levels were not noticeably

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2 E. Arner, personal communication.
decreased at the highest concentration of enzyme (200 μg/ml) (Fig. 3f), although a small amount of 374ARGSV epitope was detected (Fig. 3h). MMP-8 digestion of the 342GTRVG mutant showed that hydrolysis of the Asn 341 Phe bond was not an essential prerequisite for cleavage at Glu 373 Ala. 374ARGSV epitope was detected in digested 342GTRVG mutant at enzyme concentrations of 30 μg/ml and higher (Fig. 3l), in the absence of MMP site cleavage (Fig. 3k). The 374NVYSV mutation did not interfere with MMP-8 cleavage at DIPEN 341 FFGVG but did block cleavage at ITEGE 373 ARGSV, since there was no loss of 342FFGVG epitope at high concentrations of enzyme (Fig. 3r).

To determine whether the 342GTRVG 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 (Fig. 4). Silver staining showed that MMP-1, MMP-7, and MMP-14 cleaved the GTR mutant (Fig. 4g); however, the lack of DIPEN 341 reactivity in the digests showed that none of the MMPs cleaved at the mutated MMP site (Fig. 4f). Some 342FFGVG epitope was present in DIGSA 341 mutants digested with MMP-2, MMP-3, and MMP-14 (Fig. 4c). The data show that mutation of DIPEN 341 FFGVG to DIPEN 342 TRVG blocked cleavage at the major MMP site by three collagenases, two gelatinases, stromelysin-1, matrilysin, and MT1-MMP.

Aggrecanase Digestion of Wild-type and Mutant rG1-G2—
Wild-type and mutant rG1-G2 were incubated with increasing amounts of bovine aggrecanase, and the products were detected by Western blotting with anti-ITEGE 373 and anti-374ARGSV antibodies. All of the rG1-G2 substrates except the 374NVYSV mutant were efficiently cleaved by aggrecanase at the ITEGE 373 ARGSV site (Fig. 5). Silver staining showed that no 374NVYSV degradation products were produced by aggrecanase (Fig. 5m), suggesting that, unlike the MMPs, aggrecanase had specificity for only one site in the IGD.

Atrolysin C Digestion of Wild-type and Mutant rG1-G2—
Native glycosylated bovine aggrecan (52) and pig G1-G2 (46) are cleaved by atrolysin C at both the DIPEN 341 FFGVG and ITEGE 373 ARGSV bonds, in an independent rather than sequential manner. However, we have recently found that wild-type rG1-G2, which is largely unglycosylated, is cleaved by atrolysin C only at ITEGE 373 ARGSV and not DIPEN 341 FFGVG (46). In the present experiments, all of the rG1-G2 substrates except the 374NVYSV mutant (Fig. 6n) were cleaved by atrolysin C at ITEGE 373 ARGSV, as detected by anti-ITEGE 373 immunoreactivity (Fig. 6b, e, h, and 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 FFGVG in the wild-type, 342GTRVG, or 374NVYSV substrates. Surprisingly, 342FFGVG immunoreactivity was de-
tected in digested DIGSA\textsuperscript{241} mutant (Fig. 6f). These results suggest that the conformational shape of the substrate surrounding the sequence DIPEN\textsuperscript{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 Asn\textsuperscript{342}|Phe and Glu\textsuperscript{373}|Ala bonds, we designed experiments to test whether aggrecanase-derived G1 fragments (with ITEGE\textsuperscript{373} C terminus) were substrates for MMPs (Fig. 7) and, conversely, whether MMP-derived G2 fragments (with 342FFGVG N terminus) were substrates for aggrecanase (Fig. 8). We predicted that if aggrecanase-G1 fragments

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

**FIG. 5.** Aggrecanase digestion of wild-type and mutant rG1-G2. Recombinant G1-G2 (5 \( \mu \)g) was digested for 21 h at 37 °C with the volumes of purified aggrecanase shown, in a total volume of 20 \( \mu \)l. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analyzed by silver staining (a, d, g, j, and m) or Western blotting with anti-ITEGE\textsuperscript{373} (b, e, h, and k) or monoclonal anti-374ARGSV (c, f, i, and l).

**FIG. 6.** Atrolysin C digestion of wild-type and mutant rG1-G2. Recombinant G1-G2 (5 \( \mu \)g) was overnight at 37 °C with concentrations of atrolysin C as shown, in a total volume of 20 \( \mu \)l. Aliquots of digested and undigested rG1-G2 were electrophoresed on SDS gels and analyzed by silver staining (a, d, g, j, and m) or Western blotting with anti-ITEGE\textsuperscript{373} (b, e, h, and k) or monoclonal anti-342FFGVG (c, f, i, and l).
sequences generated by aggrecanase digestion of the MMP-G2 domain. Relative to epitope sequences generated by MMP digestion of the aggrecanase-G1 domain.

Fig. 7. Aggrecanase followed by MMP-13 sequential digest. a–g, schematic diagram showing the predicted fragments and neopeptidome sequences generated by MMP digestion of the aggrecanase-G1 domain. e–g, wild-type rG1-G2 was digested with 3 μg/ml MMP-13 for 2 h (lane 2), 3 μl of aggrecanase for 21 h (lane 3), or 3 μl of aggrecanase for 21 h followed by 3 μg/ml MMP-13 for 2 h (lane 4). Aliquots were electrophoresed on SDS gels and analyzed by Western blotting with anti-DIPEN341 (e), anti-ITEGE373 (f), or monoclonal anti-374ARGSV (g). h, the expected and observed outcome for the products obtained in d and lane 4 relative to b and lane 3 is shown.

Fig. 8. MMP-13 followed by aggrecanase sequential digest. a–d, schematic diagram showing the predicted fragments and neopeptidome sequences generated by aggrecanase digestion of the MMP-G2 domain. e–i, wild-type rG1-G2 was digested with 3 μg/ml MMP-13 for 2 h (lane 2), 3 μl of aggrecanase for 21 h (lane 3), or 3 μg/ml MMP-13 for 2 h followed by 3 μl of aggrecanase for 21 h (lane 4). e, the expected and observed outcome for the products obtained in d and lane 4 relative to b and lane 2 is shown. Aliquots were electrophoresed on SDS gels and analyzed by Western blotting with monocolonal anti-342FFGVG (f), anti-DIPEN341 (g), anti-ITEGE373 (h), or monoclonal anti-374ARGSV (i). GTRGV mutant G1-G2 was digested with 3 μg/ml MMP-13 for 2 h (lane 6), 3 μl of aggrecanase for 21 h (lane 7), or 3 μg/ml MMP-13 for 2 h followed by 3 μl of aggrecanase for 21 h (lane 8). Aliquots were electrophoresed on SDS gels and analyzed by Western blotting with monocolonal anti-374ARGSV (j).

were digested by MMPs (Fig. 7c), we would create DIPEN341 epitope, lose ITEGE373 epitope (present on the 3-kDa fragment) and observe no change in 374ARGSV epitope (Fig. 7, d and h).

The Western blots revealed that DIPEN341 epitope was indeed increased (Fig. 7e, lanes 3 and 4), while ITEGE373 epitope was completely destroyed following digestion of aggrecanase-G1 with MMP-13 (Fig. 7f, lanes 3 and 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 (Fig. 8c), we would create 374ARGSV epitope, lose 342FFGVG epitope (present on the 3-kDa fragment), and observe no change in DIPEN341 epitope (Fig. 8, d and e). However, Western blot analysis showed that 342FFGVG epitope was not destroyed (Fig. 8f, lanes 2 and 4) and that there was no gain in 374ARGSV epitope (Fig. 8i, lane 4). The small amount of anti-374ARGSV epitope present in Fig. 8i, lane 4, is most likely derived from cleavage of intact substrate rather than MMP-G2 domain, since some undigested substrate survives digestion with MMP-13 for 2 h at 3 μg/ml (Fig. 2a). Furthermore, pretreatment of rG1-G2 with MMP-13 (Fig. 8i, lane 4) markedly reduced the yield of 374ARGSV epitope by aggrecanase (compare Fig. 8i, lane 4, with Fig. 8i, lane 3). Aggrecanase was active in the presence of TIMP-inhibited MMP-13, since 342GTRVG mutants digested under the same conditions give 374ARGSV epitope (Fig. 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 IFPENFG, a substrate analogue for the MMP cleavage site, was able to inhibit the release of 374ARGSV fragments from cartilage cultured with and without interleukin-1 (SI). The results of this experiment suggested that the IFPENFG peptide was able to inhibit aggrecanase and now, in conjunction with the results presented in Fig. 8, raise 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 (Fig. 9). In these experiments, the IFPENFG 7-mer peptide was unable to block aggrecanase cleavage at Glu373 (Fig. 9, b, lane 3, and c); however, cleavage was inhibited by 50% in the presence of a 10-mer peptide with sequence FDVIFPENFFG (Fig. 9, h, lane 4, and c). A 7-mer peptide 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 (34, 36) 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 342FFGVG fragments (see Fig. 7 of accompanying article (55)) 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 342FFGVG neeoptipe is subsequently cleaved by aggrecanase, seems unlikely. Our finding that recombinant 342FFGVG fragments and native pig 342FFGVG fragments (see accompanying article (55)) 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 IITEGE373 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 342FFGVG fragments detected in synovial fluids of osteoarthritis and inflammatory arthritis patients do not contain an IITEGE373 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 cesium chloride density gradients are predominantly aggrecanase-derived and do not contain any fragments with 342FFGVG N termini (16, 17). Low buoyant density fragments fractionated on cesium chloride density gradients do contain small 342FFGVG fragments (19), and since these fragments do not carry the IITEGE373 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 accompanying article (55)); however, the detection (45) and quantitation (19) of 342FFGVG 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 IITEGE373 G1 to DIPEN341 G1 is likely to occur in vivo. Immunolocalization studies in mice with experimentally induced arthritis have shown that IITEGE373 neoepitopes were less prominent in areas showing advanced cartilage damage, compared with DIPEN341 epitope, and that when intense DIPEN341 staining appeared, IITEGE373 epitope disappeared (26). These results suggest that either IITEGE373 G1 is cleared quickly from the tissue or the G1 fragment is rapidly cleaved by MMPs or other proteinases, destroying the epitope. Under conditions where the tissue pH is acidic, the increased DIPEN341 epitope and concomitant loss of IITEGE373 epitope could arise from the endo- and exopeptidase activity of cathepsin B (53).

The peptide experiment (Fig. 9), together with our previous observation that the 7-mer peptide IPEFNF GG was able to inhibit the release of 374ARGSV fragments from cartilage in culture (51), suggests that sequences present at the C terminus of the FVDIPEN341 G1 domain may be important for aggrecanase activity. One possibility is that these sequences provide a docking site for the enzyme. If the Pro339-Glu340, or Asn339 residues were critical for aggrecanase docking or activity, we would expect that cleavage at the IITEGE373 ARGSV site may have been reduced in the DIGSA341 and deletion mutants. This was not the case, suggesting therefore that sequences other than Pro-Glu-Asn are involved. This is consistent with our observation that a longer peptide with sequence FVDIPENFFGG was effective in inhibiting aggrecanase activity by 50%, while the shorter IPEFNF GG 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 possibility could be addressed by determining whether a peptidic hydroxamate inhibitor based on the Pro-Glu-Asn residues could inhibit aggrecanase.

Three MMP cleavage site mutants were made, and these partially or totally blocked cleavage at the DIPEN341FFGVG 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 342GTRVG mutant resisted cleavage by stromelysin-1, two gelatinases, three collagenases, matrilysin, and MT1-MMP at the mutated MMP site. The DIGSA341 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 374NVYSV 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 374NVYSV 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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