Membrane type 1 matrix metalloproteinase (MT1-MMP) cleaves the recombinant aggrecan substrate rAgg1\textsubscript{mut} at the ‘aggrecanase’ and the MMP sites

Characterization of MT1-MMP catabolic activities on the interglobular domain of aggrecan

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The recent detection of membrane type 1 matrix metalloproteinase (MT1-MMP) expression in human articular cartilage [Büttnner, Chubinskaya, Margerie, Huch, Flechtenmacher, Cole, Kuettner, and Bartnik (1997) Arthritis Rheum. 40, 704–709] prompted our investigation of MT1-MMP’s catabolic activity within the interglobular domain of aggrecan. For these studies we used rAgg1\textsubscript{mut}, a mutated form of the recombinant fusion protein (rAgg1) that has been used as a substrate to monitor ‘aggrecanase’ catabolism in vitro [Hughes, Büttnner, Eidenmüller, Caterson and Bartnik (1997) J. Biol. Chem. 272, 20269–20274]. The rAgg1 was mutated (G272 to A) to avoid the generation of a splice variant seen with the original genetic construct, which gave rise to heterogeneous glycoprotein products. This mutation yielded a homogeneous recombinant product. Studies in vitro with retinoic acid-stimulated rat chondrosarcoma cells indicated that the rAgg1\textsubscript{mut} substrate was cleaved at the ‘aggrecanase’ site equivalent to Glu\textsuperscript{373}-Ala\textsuperscript{374} (human aggrecan sequence enumeration) in its interglobular domain sequence segment.

The differential catabolic activities of the recombinant catalytic domain (cd) of MT1-MMP and matrix metalloproteinases (MMPs) 3 and 8 were then compared by using this rAgg1\textsubscript{mut} as a substrate. Coomassie staining of rAgg1\textsubscript{mut} catabolites separated by SDS/PAGE showed similar patterns of degradation with all three recombinant enzymes. However, comparative immunodetection analysis, with neoepitope antibodies BC-3 (anti-ARGS…), BC-14 (anti-FFGV…), and chondroitinase ABC, indicated that the catalytic domain of MT1-MMP exhibited strong ‘aggrecanase’ activity, cdMMP-3 weak activity and cdMMP-3 no activity. In contrast, cdMMP-3 and cdMMP-8 led to strongly BC-14-reactive catabolic fragments, whereas cdMT1-MMP resulted in weak BC-14 reactivity. N-terminal sequence analyses of the catabolites confirmed these results and also identified other potential minor cleavage sites within the interglobular domain of aggrecan. These results indicate that MT1-MMP is yet another candidate for ‘aggrecanase’ activity in vivo.

INTRODUCTION

Articular cartilage covering the end of the bones in synovial joints consists of a highly organized extracellular matrix, produced and maintained by chondrocytes. The extracellular matrix, consisting of a dense collagen network embedded in a proteoglycan gel, is necessary and responsible for the compressive resilience of the cartilage tissue. In human arthritis the loss of aggrecan, the major proteoglycan in cartilage, leads to joint damage and with this to loss of joint function. The normal cartilage turnover and the pathological loss of aggrecan involves a predominant proteolytic cleavage in the interglobular domain (IGD) of the aggrecan core protein [1–4]. N-terminal amino acid sequence analyses of proteoglycan metabolites isolated from synovial joint fluid samples of patients with joint injury, osteoarthritis and inflammatory joint diseases indicate that proteolytic cleavage occurs between Glu\textsuperscript{373} and Ala\textsuperscript{374} (human sequence enumeration [5]) within the IGD of human aggrecan [6,7]. Similarly, studies in vitro with cartilage explants or chondrocyte cultures exposed to cytokines or retinoic acid have also identified Glu\textsuperscript{373} and Ala\textsuperscript{374} as the major site of proteolytic cleavage in the IGD of aggrecan [1,8]. This unknown activity denoted as ‘aggrecanase’ is thought to have a central role in the catabolism of cartilage aggrecan in joint pathology.

Many matrix metalloproteinases (MMPs) have been shown to cleave the aggrecan IGD at the Asn\textsuperscript{341}-Phe\textsuperscript{342} bond [9–12]; the G1 domain generated by this cleavage has been isolated from human cartilage [11]. Stromelysin-1 (MMP-3) [10], neutrophil collagenase (MMP-8) [13] and collagenase-3 (MMP-13) [14] are all known to degrade aggrecan in vitro at this site. In addition, whereas neutrophil collagenase (MMP-8) was shown to cleave the Asn\textsuperscript{341}-Phe\textsuperscript{342} bond preferentially [12,15], it can also cleave the Glu\textsuperscript{373}-Ala\textsuperscript{374} bond after a prolonged incubation of the aggrecan G1-G2 fragment (the IGD region of human aggrecan, containing the globular domains G1 and G2) at high enzyme concentrations [15], implying that it might be a candidate for ‘aggrecanase’ activity seen in vivo [16]. These results suggest that MMPs are involved in aggrecan degradation; however we are still uncertain of the identity of ‘aggrecanase’ and also which of the MMPs in cartilage has the central role in this process. Multiple cleavage sites of ‘aggrecanase’ and individual MMPs along the core protein of aggrecan further complicate exper-

Abbreviations used: cd, catalytic domain; G1-G2, IGD region of human aggrecan containing the globular domains G1 and G2; IGD, interglobular domain; MALDI, matrix-assisted laser desorption ionization; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; rAgg1, recombinant aggrecan fusion protein 1; rAgg1\textsubscript{mut}, recombinant mutated aggrecan fusion protein 1.

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imental approaches aimed at improving our understanding of these catabolic pathways.

Recently we described the use of a recombinant IGD substrate to study aggrecan catabolism by ‘aggrecanase’ in cell culture systems in vitro [17]. The use of this recombinant polypeptide substrate (rAgg1) considerably simplified methods needed to identify aggrecan IGD catabolites and has afforded us a useful reagent for studying the actions of other catabolic agents on the IGD of aggrecan.

Our recent finding that membrane type 1 matrix metalloproteinase (MT1-MMP) is expressed in human articular cartilage [18], together with the reports that MT1-MMP activates gelatinase A (MMP-2), MMP-13 and proteolyses extracellular matrix components including aggrecan [19–22] and the speculation by Ohuchi et al. [22] that ‘aggrecanase’ activity might be MT1-MMP or a combined action of MT1-MMP and MMP-2, led us to examine whether or not MT1-MMP catalyzes aggrecan catabolism directly and, if so, at which site(s) in the aggrecan IGD. In the present study we found that the catalytic domain (cd) of MT1-MMP cleaves at multiple sites within the IGD of the fusion protein rAgg1mut, including the major and minor MMP cleavage sites and most notably at the ‘aggrecanase’ site. Although less pronounced, cdMMP-8 cleaves rAgg1mut also at the ‘aggrecanase’ site and, like cdMMP-3, at additional sites including the major MMP site [15,23].

**EXPERIMENTAL**

**Materials**

N-glycosidase F, O-glycosidase, neuraminidase, Lys C endopeptidase and alkaline phosphatase-conjugated anti-mouse antibody were all obtained from Boehringer Mannheim (Mannheim, Germany). The alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate and alkaline phosphatase-conjugated anti-mouse antibody were all obtained from Boehringer Mannheim. Monoclonal antibodies conjugated anti-(human IgG) and anti-mouse antibody were all obtained from Boehringer Mannheim. Protein A (MMP-2), MMP-13 and proteolyses extracellular matrix proteins including aggrecan [19–22] and the speculation by Ohuchi et al. [22] that ‘aggrecanase’ activity might be MT1-MMP or a combined action of MT1-MMP and MMP-2, led us to examine whether or not MT1-MMP catalyzes aggrecan catabolism directly and, if so, at which site(s) in the aggrecan IGD. In the present study we found that the catalytic domain (cd) of MT1-MMP cleaves at multiple sites within the IGD of the fusion protein rAgg1mut, including the major and minor MMP cleavage sites and most notably at the ‘aggrecanase’ site. Although less pronounced, cdMMP-8 cleaves rAgg1mut also at the ‘aggrecanase’ site and, like cdMMP-3, at additional sites including the major MMP site [15,23].

**Synthesis and characterization of rAgg1mut**

For PCR cloning, the following forward and reverse primers were used: forward primer: 5'-CCG GGG GTA GCC GAC TAC AAG GAC GAC GAT GAC AAG ACC GCA GAA GAC TTT GTG GAC ATC CCA-3'; reverse primer: 5'-CG CGG GGG ATC CCC TCC CCC TCC TGG CAA ATG CGG CTG CCC-3'. To eliminate an alternative splice donor site from the N-terminal sequence coding for the IGD of aggrecan [17], a mutation was incorporated in the forward primer, changing Gly122 of the aggrecan IGD sequence into Ala. ThePCR product was digested with NheI and BamHI and ligated into NheI and BamHI-cleaved CD5-IgG vector [26]. The correct sequence of the rAgg1mut construct was verified by Southern sequencing. The expression in COS-7 cells and the affinity purification of rAgg1mut were performed as described previously [17]. For N-terminal sequence analyses, limited proteolysis of 20 µg of rAgg1mut was done in 50 µl of 25 mM Tris/HCl (pH 8.5)/1 mM EDTA with 2 µg of Lys C protease for 18.5 h at 30 °C and stopped by the addition of 3 µl of 25 % (v/v) trifluoracetic acid. Peptide fragments were subsequently resolved by HPLC and their N-terminal amino acid sequences were analysed. Matrix-assisted laser desorption ionization (MALDI) MS of rAgg1mut was performed on a Biflex spectrograph (Bruker, Germany).

For enzymic deglycosylation, 10 µg of affinity-purified freeze-dried rAgg1mut was dissolved in 10 µl of denaturation solution [1 % (w/v) SDS/10 % (v/v) 2-mercaptoethanol] and boiled for 2 min. After the addition of 90 µl of incubation buffer [20 mM sodium phosphate (pH 7.2)/50 mM EDTA (pH 8.1)/10 mM sodium azide/0.5 % octylglycoside] the solution was boiled again for 2 min. After equilibration to 37 °C, 0.4 unit of N-glycosidase F, 2.5 m-units of O-glycosidase and 2.0 m-units of neuraminidase were added and then incubated at 37 °C for 18 h. Digests were then analysed by SDS/PAGE as described below.

**Rat chondrosarcoma cell culture and catabolism of the rAgg1mut substrate**

Rat chondrosarcoma Rx cells (kindly provided by Dr. James Kimura, Henry Ford Hospital, Detroit, MI, U.S.A.) were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium (Gibco) with 50 µg/ml gentamycin (Gibco), 2 mM glutamine (Gibco) and 5 % (v/v) fetal bovine serum (Sigma). At confluence, cells were harvested with treatment with trypsin/EDTA (Gibco) and cultured in agarose (with or without 1 µM retinoic acid) in 24-well plates (4 × 10^5 cells per well) under conditions described previously [17]. To study the catabolism of rAgg1mut fusion protein, 50 µg of the protein was added to the agarose suspension in each well and incubated for 72 h at 37 °C. Culture medium was collected individually, dialysed overnight against water and freeze-dried, then analysed by SDS/PAGE and immunodetection with antibody BC-3.

**Cloning, expression and purification of cdMMP-3, cdMMP-8 and cdMT1-MMP**

All catalytic domains of MMPs were obtained through PCR cloning of the corresponding cDNA species and insertion of the respective cDNA species into the T7 expression vector pET11a (Novagen) and isopropyl β-D-thiogalactoside-induced protein expression in the *Escherichia coli* strain BL21(DE3) [27]. Genetic constructs for cdMMP-3 encompassed the catalytic domain, ranging from Gly98 to Pro173 [28], and for cdMMP-8 the catalytic domain, ranging from Gly97 to Gin266[13]. Cloning, expression, protein purification and refolding was done for both proteins in accordance with published methods [29]. Genetic constructs for both proteins provided an additional Met residue as the N-terminal amino acid. cdMT1-MMP was derived from a full-length clone [18] and the recombinant enzyme encompassed the correctly folded propeptide and the catalytic domain, ranging from Ala21 to Ile318 [30].

**Catabolism of rAgg1mut by cdMMP-3, cdMMP-8 and cdMT1-MMP**

Experiments involving the catabolism of rAgg1mut [312.5 µg/ml (6 µM)] were performed in buffer containing 10 mM CaCl\(_2\), 100 mM NaCl and 50 mM Tris/HCl, pH 7.5, at 37 °C for 17 h. The cdMMP concentrations used were 10, 40, 100 and 180 µg/ml. The given concentrations correspond to 0.52, 2.1, 5.2 and 9.4 M for cdMT1-MMP. Concentrations of cdMT1-MMP of 3–100 µg/ml (0.13–4.5 µM) were used to titrate the catalytic activity of cdMT1-MMP on the rAgg1mut substrate. To prevent non-specific cleavage, recombinant substrate and enzymes were stored with 1 mM PMSF/2 µM pepstatin/56 µM *trans*-epoxysuccinyl-l-tyr(4-guanidino)butane (E-64). These inhibitors had no influence on the rAgg1mut catabolism experiments.
Semi-D/3PAGE, Western blotting and immunostaining

Purified rAgg1mut protein, deglycosylated rAgg1mut, dialysed and freeze-dried experimental cell-culture medium containing catalyzed rAgg1mut substrate and also rAgg1mut derived from the cdMMP catabolism experiments, were all fractionated by SDS/PAGE [10% (w/v) gel] [31] and transferred to PVDF membranes. For immunodetection, antibody BC-3 and BC-14 ascites solutions were diluted 1:1000 and used as described previously [17,24,25]. For N-terminal amino acid sequence analysis, polypeptides were transferred to PVDF membranes, Coomassie stained and subjected to Edman degradation [32].

RESULTS

Characterization of rAgg1mut

For previous studies aimed at characterizing ‘aggrecanase’ activity, we developed the recombinant fusion protein rAgg1, which was cleaved at the A$(%RGS site in cultures of the rat chondrosarcoma cell line Rx and primary bovine chondrocytes after stimulation with retinoic acid [17]. The essential feature of the rAgg1 recombinant fusion protein was that it contained the complete IGD polypeptide sequence of the human proteoglycan aggrecan. As reported, transient expression of this fusion protein in COS-7 cells leads to the synthesis of soluble proteins, which can be resolved on reducing SDS/PAGE gels as two components of 39 and 72 kDa [17]. N-terminal sequence analysis of both bands indicated that the 39 kDa component resulted from the partial functionality of an alternative splice donor site, contained in the nucleotide sequence corresponding to the N-terminal end of the IGD of the human aggrecan gene [5,33]. To prevent this alternate splicing and to simplify work with this recombinant substrate, the splice donor site was deleted. PCR cloning with an oligonucleotide coding for a mutated N-terminus of the IGD, where Gly322 was mutated into Ala, was used to create the new rAgg1mut genetic construct. This mutated construct served for the expression of rAgg1mut, used for the studies described here. Similarly to its parent construct [17], the rAgg1mut contains a signal sequence derived from the leucocyte cell-surface protein CD5 to facilitate expression, the FLAG epitope [34] as an N-terminal tag, the IGD of the human aggrecan ranging from Thr331 to Gly458 [5], a four-residue spacer and the constant region of the heavy chain of a human IgG molecule as a C-terminal tag, which includes the hinge, CH2 and CH3 domains. On transient expression in COS-7 cells, rAgg1mut is secreted into the culture supernatant with a yield of 2–4 µg ml of culture supernatant.

After affinity purification on anti-FLAG M1 antibody columns and resolution by SDS/PAGE, rAgg1mut showed a single band with an apparent molecular mass of 72 kDa under reducing conditions (Figure 1, lane 3). No evidence of the 39 kDa product from the alternative splice variant was present in this preparation. Non-reducing SDS/PAGE gels showed a prominent band of 140 kDa and, in addition, higher-molecular-mass bands (Figure 1, lane 2), reflecting dimerization and multimerization products due to an unpaired cysteine in the hinge region of the human immunoglobulin component of the rAgg1mut protein. Similar aggregates (under non-reducing conditions) were also present in its parent molecule rAgg1 [17]. Limited proteolysis of rAgg1mut with Lys C protease and subsequent HPLC yielded peptide fragments that, after N-terminal amino acid sequence analysis, verified the correct expression of amino acid sequences comprising components (Figure 2) of the rAgg1mut construct.

To resolve the discrepancy between the predicted and observed molecular masses (41 kDa and 72 kDa respectively) of rAgg1mut, purified protein was subjected to MALDI-MS, which resolved a
major peak at 52 kDa. The MALDI signal for rAgg1mut was resolved as a broad peak, probably owing to variable glycosylation, thus enabling only an approximate mass determination. Deglycosylation with N-glycosidase, O-glycosidase and neuraminidase shifted the size of rAgg1mut on reducing SDS/PAGE gels from 72 kDa to approx. 62 kDa (Figure 1, lanes 4 and 5), indicating that rAgg1mut expression in COS cells yields proteins whose mass is increased by approx. 10 kDa by glycosylation; this mass increase was also verified by means of chemical carbohydrate analysis. This analysis also showed that sugar residues of the rAgg1mut protein expressed in COS cells are both N- and O-linked oligosaccharides formed by complex and/or high-mannose types. Only one or two of the potential N-glycosylation sites found in the amino acid sequence of the rAgg1mut protein (Asn14, Asn38, Asn39; see Figure 2) are glycosylated. The different use of the three potential glycosylation sites could be the reason for the observed variable glycosylation pattern. The calculated molecular mass of 41 kDa and a mass of 10 kDa glycosylation compare favourably with the overall molecular mass of approx. 52 kDa, observed by MALDI analysis of rAgg1mut. The observed migration at 72 kDa on SDS/PAGE gels might result from the binding of less SDS to this recombinant protein.

‘Aggrecanase’ catabolism of rAgg1mut

To test whether rAgg1mut was an accepted substrate for ‘aggrecanase’ catabolism, rAgg1mut was added to rat chondrosarcoma Rx cells cultured in agarose [17]. On stimulation with 1 µM retinoic acid, Rx cells produce ‘aggrecanase’ activity [8,17]. After 72 h of stimulation, cell culture medium was dialysed, freeze-dried and taken up in sample buffer for SDS/PAGE and Western blotting analysis. Immunodetection with the A7.2RGS… neoepitope-specific antibody BC-3 [24] detected a 66 kDa band in the culture medium of stimulated cells (Figure 1, lane 7) that was absent from the medium of unstimulated cells (Figure 1, lane 6). The size (66 kDa) of this catabolic product is in agreement with the predicted loss of a 5.8 kDa polypeptide after cleavage at the ‘aggrecanase’ site and similar to that observed with the rAgg1 parent molecule [17].

Cleavage of rAgg1mut by cdMMP-3, cdMMP-8 and cdMT1-MMP

The reports that MT1-MMP cleaved aggrecan [21,22] and our observation of MT1-MMP expression in human chondrocytes [18] led us to investigate whether MT1-MMP could cleave the recombinant substrate rAgg1mut at the ‘aggrecanase’ site. Because neutrophil collagenase (MMP-8), which is also expressed in cartilage [16], is already known to cleave a G1-G2 fragment of aggrecan at the ‘aggrecanase’ site at high enzyme concentrations (160 µg/ml) [15], we examined the catabolic activities of cdMMP-3, cdMMP-8 and cdMT1-MMP on the recombinant IGD substrate rAgg1mut. Recombinant catalytic domains of these enzymes were incubated at 10, 40 or 180 µg/ml with 312.5 µg/ml rAgg1mut for 17 h at 37 °C. The given concentrations correspond to 0.52, 2.1 and 9.4 µM for cdMMP-3, 0.53, 2.1 and 9.5 µM for cdMMP-8, 0.45, 1.8 and 8.1 µM for cd MT1-MMP, and 6 µM for rAgg1mut. Figure 3(A) shows the Coomassie staining of uncatalysed rAgg1mut (lane 1) and the resulting recombinant enzyme cleavage products (lanes 2–10) separated on reducing SDS/PAGE gels. The staining patterns for the proteins and polypeptides show distinct similarities and differences. As seen in lanes 2–4, cdMMP-3 cleavage of rAgg1mut created fragments of 35 kDa and of 42 kDa, and at 180 µg/ml enzyme concentration a band of approx. 66 kDa (lane 4). cdMMP-8 catabolized rAgg1mut into two fragments of 66 and 42 kDa (lanes 5–7). A
163

Membrane type 1 matrix metalloproteinase and ‘aggrecanase’ cleavage

Table 1 N-terminal peptide sequences of rAgg1mut catabolized by cdMMPs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular mass of analysed bands (kDa)</th>
<th>N-terminal amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>cdMMP-3</td>
<td>66</td>
<td>F20FGVXXXXXQDXXVXTXPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I47TEGEAXSXVILTVKPIFE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S55VILTVKPIFEVXXPLE</td>
</tr>
<tr>
<td>cdMMP-8</td>
<td>66</td>
<td>F20FGVXXXXXQDXXVXTXPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I47TEGEAXSXVILTVKPIFE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S55VILTVKPIFEVXXPLE</td>
</tr>
<tr>
<td>cdMT1-MMP</td>
<td>72</td>
<td>F20FGVXXXXXQDXXVXTXPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I47TEGEAXSXVILTVKPIFE</td>
</tr>
<tr>
<td></td>
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<td>S55VILTVKPIFEVXXPLE</td>
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</tbody>
</table>

very similar fragmentation pattern was also seen after cleavage with cdMT1-MMP (lanes 8–10). To identify the cleavage products, polypeptides were transferred to PVDF membranes and subjected to N-terminal sequence analysis. Table 1 summarizes the amino acid sequence analyses of these fragments. The occurrence of multiple sequences within bands is probably due to insufficient resolution of peptide fragments on the SDS/PAGE gels. These results indicate multiple cleavage sites in the rAgg1mut substrate, several of these at known MMP cleavage sites (see below).

Neoepitope-specific antibodies BC-3 and BC-14 were employed for immunochemical analysis of the cleavage products shown in Figure 3(A); the immunoblots are depicted in Figure 3(B). Monoclonal antibody BC-14 [25] specifically reacts with the neoepitope FFGV..., reflecting a cleavage at the major MMP catabolic site in the aggrecan IGD [9–12], whereas BC-3 monoclonal antibody detects a cleavage at the ‘aggrecanase’ site within the IGD [24].

As shown in Figure 3(B), cdMMP-3 cleavage of rAgg1mut produced no BC-3-reactive fragments (lane 1), whereas rAgg1mut catabolized with cdMMP-8 resulted in a weak BC-3 signal (lane 2). cdMT1-MMP cleavage of rAgg1mut resulted in a strong BC-3 signal (lane 3). Both cdMMP-3 and cdMMP-8 cleavage produced fragments strongly reactive with BC-14 (lanes 4 and 5), whereas cleavage with cdMT1-MMP gave only a weak BC-14 reactivity (lane 6). Enzymes were used in comparable molar concentrations: cdMMP-3 at 5.2 µM, cdMMP-8 at 5.3 µM and cdMT1-MMP at 4.5 µM.

To evaluate the different apparent efficacy of cdMMP-8 and cdMT1-MMP cleavage as indicated by the difference in BC-3 reactivities in Figure 3(B) (compare lanes 2 and 3), we performed a time course of rAgg1mut cleavage with cdMT1-MMP (at 4.5 µM) and cdMMP-8 (at 5.3 µM) as shown in Figure 3(C). With cdMT1-MMP a prominent BC-3 reactivity was seen after only 15 min of incubation, whereas cdMMP-8 produced a similar signal only after 120 min of incubation. A titration of cdMT1-MMP, as shown in Figure 3(D), produced BC-3 reactive fragments of rAgg1mut even at a concentration of 3 µg/ml (0.13 µM), corresponding to a 46-fold molar excess of substrate over enzyme.

The finding of an ‘aggrecanase’-generated cleavage with cdMT1-MMP was verified by detecting the sequence ARGS... in the 66 kDa fragment after cleavage with MT1-MMP, by using amino acid sequencing (see Table 1). A summary of the results in Figure 3 and Table 1 is illustrated in Figure 4. The cleavage patterns resulting from the catabolism of rAgg1mut substrate by cdMMP-3 and cdMMP-8 are similar to those reported in the literature when native aggrecan or aggrecan G1-G2 fragments were used as substrates [9,10,12,15]. In addition we have identified some additional sites of potential proteolytic cleavage within the IGD of aggrecan.

DISCUSSION

The central finding of this paper is the experimental evidence that the catalytic domain of MT1-MMP cleaves the recombinant aggrecan fusion protein rAgg1mut at the ‘aggrecanase’ site corresponding to Glu$^{372}$-Ala$^{374}$ of native aggrecan, even at low enzyme concentrations (3 µg/ml; 0.13 µM), or at a 46-fold molar excess of substrate over enzyme. cdMMP-8 was also found to
cleave at the aggrecanase site of rAgg1mut but with much less efficacy than cdMT1-MMP. No cleavage activity could be detected for cdMMP-3 at the aggrecanase site.

With this finding, MT1-MMP becomes, in addition to MMP-8 and Atrolysin C, a member of the ADAM family [35], one more candidate enzyme that potentially explains ‘aggrecanase’ activity seen in vitro [6, 7]. This finding supports the speculation of Ohuchi et al. [22] that ‘aggrecanase’ might be MT1-MMP. The absence of any other MMP in our system in vitro possibly rules out the second speculation by Ohuchi et al. that ‘aggrecanase’ might be a combined action of MT1-MMP and MMP-2, but MMP-2 could nevertheless be responsible for the processing of full-length MT1-MMP.

MMP-8 was described by Fosang et al. [15] as the first enzyme to cleave at the major ‘aggrecanase’ site (Glu172-Ala174) in the IGD of aggrecan. However, they found, by using a G1-G2 fragment of aggrecan as a substrate, that this ‘aggrecanase’-type cleavage occurs only at high enzyme concentrations (160 µg/ml) or after the addition of 4 kDa poly(ethylene glycol) (PEG 4000), which increases the local enzyme concentration. The predominant cleavage site for MMP-8 in the IGD of aggrecan is the major MMP-cleavage site occurring between Asn134 and Phe412, whereas the Glu172-Ala174 site is less preferred [15, 23]. As we observed cleavage at the aggrecanase site by cdMMP-8 only at the much higher enzyme concentration of 100 µg/ml (5.3 µM) and after a longer incubation than needed with cdMT1-MMP, we argue that cdMMP-8 cleaves with lower efficacy at the aggrecanase site of rAgg1mut than does cdMT1-MMP. For comparison, MMP-3 and MMP-1 occur in the synovial fluid from patients with arthritis in concentrations ranging from 2 to 40 µg/ml [36, 37]. Lichte et al. [30] used, with the same cdMT1-MMP as in this study, encompassing Ala211-Ile218, a 4-fold molar excess of substrate over enzyme to activate pro-gelatinase A and pro-gelatinase B with TIMP-2.

Fosang et al. [15] and Arner et al. [23] reported that MMP-8 cleaves at the aggrecanase site by using G1-G2 aggrecan and native full-length aggrecan. Our findings are in agreement with these reports, because cdMMP-8 also cleaved at the aggrecanase site provided in our recombinant rAgg1mut fusion protein. From this we conclude that rAgg1mut is a useful substitute for native aggrecan because all of the cleavage sites known to occur on native aggrecan are also found on rAgg1mut substrate.

The applicability of rAgg1mut as a general substrate for MMPs was specifically shown with its degradation by cdMMP-3, cdMMP-8 and cdMT1-MMP. As illustrated schematically in Figure 4, MMPs cleave at multiple sites within the IGD of human aggrecan [9–12, 14]. rAgg1mut is also cleaved by MMPs at multiple sites. Some of the sites correspond to known cleavage sites on native aggrecan: all three enzymes cleaved at the major MMP site (Asn134-Phe412 of native aggrecan) and MT1-MMP also cleaved at the minor MMP site (Asp141-Leu414 of native aggrecan) and at Ser235-Val236 (Ser237-Val238 of native aggrecan), which was also previously observed for MMP-3 [15]. In addition, our results indicate several novel activities on the rAgg1mut substrate with cleavages C-terminal to Asn96, Gly94, Ala113, Phe115 and Pro156. There are several possible explanations of these novel cleavages. (1) Our system in vitro with a purified recombinant substrate might offer a higher sensitivity than other assay systems with substrates derived from native aggrecan. (2) Catalytic domains might convey catalytic activities different from those of full-length enzymes. However, other studies indicate that catalytic domains or truncated forms of enzymes usually cleave at fewer, rather than more, sites along the substrate (see, for example, [38]). (3) Differences in secondary structure or post-translational modifications between the native and the recombinant substrate might render rAgg1mut susceptible to cleavages at sites other than those in the native substrate.

The observed novel cleavage site between Asn46 and Ile54 in the rAgg1mut substrate, corresponding to a cleavage between Asn66 and Ile669 of human aggrecan, could explain the findings of Fosang et al. [39], who described in their studies IGD fragments that possessed the FFG N-termini but lacked the ITEGE C-termini. Similarly our finding supports the hypothesis of Arner et al. [23] that MMP-8 cleaves preferentially at another site, probably the MMP site, and at an additional site towards the C-terminus of aggrecan, before cleaving at the Glu172-Ala174 bond. The sensitivity of our system in vitro even permitted the detection of further cleavages at Gly35-Ser35 (Gly37-Ser37) and Ala114, Phe115 (Ala116-Phe117), although the relevance to cleavage sites in native aggrecan in vivo has yet to be established.

The exact pathway of the proteolytic breakdown of aggrecan is still under debate [8, 39]. Lark et al. [40] detected MMP-generated G1 fragments terminating in VDIPEN394 and aggrecanase-generated G1 fragments terminating in NITEGE376 in various cartilage samples and thus provide evidence for both MMP and aggrecanase activity in normal, osteoarthritic and rheumatoid joints. From their observation that in some regions of the tissue both neoepitopes were found, whereas in others only one was detected, they concluded that the generation and/or turnover of these specific catabolic fragments is not necessarily co-ordinated. As our studies indicate, cdMT1-MMP is able to cleave rAgg1mut at multiple sites: the site corresponding to the major MMP cleavage site on human aggrecan, the aggrecanase site, between Ser35 and Val36 (Ser37-Val38) and the minor MMP cleavage site. Thus cdMT1-MMP could have the role of an enzymic activity able to perform multiple catabolic events independently of each other. The determination at what particular site the actual cleavage would occur might depend on the physical environment and substrate presentation of the individual substrate molecule, as discussed by Arner et al. [23]. In addition other features of MT1-MMP correlate with the detection of G1 fragments in cartilage from normal donors and in cartilage of patients of different ages [40]. Those features are its expression in normal and osteoarthritic cartilage and its expression in cartilage of donors of different age ([41], and F. H. Büttner, S. Chubinskaya, A. Cole and E. Bartnik, unpublished work). Additionally, the potential localization of MT1-MMP either close to the cell surface as a transmembrane protein or in the matrix as a soluble enzyme correlates with both detections of G1 fragments in the vicinity of cells and further removed in the interterritorial matrix [40].

The mutation of the alternative splice site in the rAgg1mut construct resulted in the expression of a protein giving rise to a distinct, although broad, band on SDS/PAGE gels. This feature can be explained by variable glycosylation at the three expected glycosylation sites within the IGD of rAgg1mut (Asn66, Asn96 and Asn237). High-efficiency transient expression systems such as the COS system employed are known to generate highly variable glycosylation patterns on overexpressed proteins.

The mutation of the alternative splice site in the rAgg1mut construct did not change the apparent molecular mass of the recombinant protein of 72 kDa towards the predicted mass of 42 kDa. The reason for this might be incomplete complexing with SDS and/or structural specifics of rAgg1mut. This applies not only to the full-length recombinant protein but also to proteolytic fragments of rAgg1mut. This might explain why products with an N-terminus of Thr116 or Leu119 (see Table 1) show an apparent electrophoretic size of approx. 42 kDa instead of a predicted size of 28 kDa.

In summary, our paper provides the following evidence that rAgg1mut is a useful substitute for native aggrecan in studying...
catabolic events in the IGD of aggrecan very sensitively: (1) aggrecanase from Rx cell cultures accepts rAgg1mut as a substrate; (2) all known MMP cleavage sites of native aggrecan were found with rAgg1mut, and (3) novel cleavage sites (i.e. at Asn\textsuperscript{XaaY}) can explain the catabolic fragments detected. With the activity of MMP-8, MT1-MMP and Atrolysin C [35], the first two known enzymes (and others) that 'aggrecanase' proteolysis found in the IGD of aggrecan very sensitively: (1) the 'aggrecanase' site occurs only if multiple parameters are fulfilled. Those parameters might include the substrate aggrecan (i.e. at Asn\textsuperscript{XaaY}), the notion becomes stronger that 'aggrecanase' proteolysis found in the IGD of aggrecan very sensitively: (1) the proteases can be expressed in cartilage [18,41], the notion becomes stronger that 'aggrecanase' proteolysis found in the IGD of aggrecan very sensitively: (1) the environmental conditions (pH, ionic conditions and cofactors) plus one of several suitable, possibly also modified, proteases able to execute the cleavage.

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**REFERENCES**


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