Recombinant Human Aggrecan G1-G2 Exhibits Native Binding Properties and Substrate Specificity for Matrix Metalloproteinases and Aggrecanase*

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A recombinant human aggrecan G1-G2 fragment comprising amino acids Val1–Arg556 has been expressed in Sf21 cells using a baculovirus expression system. The recombinant G1-G2 (rG1-G2) was purified to homogeneity by hyaluronan-Sepharose affinity chromatography followed by high performance liquid chromatography gel filtration, and gave a single band of Mr 90,000–95,000 by silver stain or immunoblotting with monoclonal antibody 1-C-6. The expressed G1-G2 bound to both hyaluronan and link protein indicating that the immunoglobulin-fold motif and proteoglycan tandem repeat loops of the G1 domain were correctly folded. Further analysis of secondary structure by rotary shadowing electron microscopy confirmed a double globe appearance, but revealed that the rG1-G2 was more compact than its native counterpart. The size of rG1-G2 by SDS-polyacrylamide gel electrophoresis was unchanged following digestion with keratanase and keratanase II and reduced by only 2–5 kDa following digestion with either O-glycosidase or N-glycosidase F. Recombinant G1-G2 was digested with purified matrix metalloproteinases (MMP), isolated aggrecanase, purified atrolysin C, or proteinases present in conditioned medium from cartilage explant cultures, and the products analyzed on SDS gels by silver stain and immunoblotting. Neopeptide antibodies recognizing the N-terminal F342FGVG or C-terminal DIPEN341 sequences were used to confirm MMP cleavage at the Asn341 ↓ Phe bond, while neopeptide antibodies recognizing the N-terminal A374RGSV or C-terminal ITGE373 sequences were used to confirm aggrecanase cleavage at the Glu373 ↓ Ala bond. Cleavage at the authentic MMP and aggrecanase sites revealed that these proteinases have the same specificity for rG1-G2 as for native aggrecan. Incubation of rG1-G2 with conditioned medium from porcine cartilage cultures revealed that active soluble aggrecanase but no active MMPs, was released following stimulation with interleukin-1α or retinoic acid. Atrolysin C, which cleaves native bovine aggrecan at both the aggrecanase and MMP sites, efficiently cleaved rG1-G2 at the aggrecanase site but failed to cleave at the MMP site. In contrast, native glycosylated G1-G2 with or without keratanase treatment was cleaved by atrolysin C at both the aggrecanase and MMP sites. The results suggest that the presence or absence per se of keratan sulfate on native G1-G2 does not affect the activity of atrolysin C toward the two sites.

Aggrecan is a chondroitin sulfate and keratan sulfate-bearing proteoglycan. It is present in cartilage as large multimolecular aggregates bound noncovalently to hyaluronan (HA).1 Aggregate formation is mediated via the proteoglycan tandem repeat loops in the N-terminal G1 domain which bind to deacetylated HA in the polymeric HA. This binding is further stabilized by link protein, which binds to form stable trimeric complexes. The formation of large aggregate structures provides a mechanism for trapping aggrecan in the tissue and preventing its loss by diffusion. A major feature of the pathology of arthritis is the gradual loss of aggrecan from the cartilage matrix. This loss involves proteolysis of the core protein and release into culture medium (1, 2) or synovial fluid (3) of large fragments lacking G1 domains that are therefore unable to bind hyaluronan. The interglobular domain (IGD) between G1 and G2 is particularly sensitive to proteolysis although there is catalytic processing of more C-terminal regions of aggrecan as well.

Following cloning and sequencing of the full-length cDNA for human aggrecan (4), sequence analysis of aggrecan degradation products revealed the location of specific aggrecanase cleavage sites within the core protein (5–7). The Glu373 ↓ Ala aggrecanase site within the IGD has been most widely studied since cleavage at this site releases the entire glycosaminoglycan-bearing portion of the molecule and therefore has the greatest biological consequence for weight bearing functions. The N-terminal sequence of the IGD-derived aggrecanase fragment found in cartilage explant medium was ARGSV, commencing at Ala374 (5–7) (Fig. 1). Aggrecan fragments with the same A374RGSV N termini have since been identified in synovial fluids from osteoarthritis patients (8) and patients with inflammatory arthritides and joint injury (9) indicating that

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1 The abbreviations used are: HA, hyaluronan; G1, first (N-terminal) globular domain of aggrecan; G2, second globular domain of aggrecan; IGD, interglobular domain separating the G1 and G2 globular domains; MMP, matrix metalloproteinase; GdnHCl, guanidinium hydrochloride; IL-1α, interleukin-1α; bp, base pairs; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TIMP, tissue inhibitor of metalloproteinase.
Aggrecanase is a key enzyme in human aggrecan catabolism. An extensive literature over several decades has proposed that matrix metalloproteinases (MMPs) are directly involved in aggrecan degradation based on circumstantial evidence that levels of several MMPs are elevated in arthritic disease and that aggrecan release from cartilage can be blocked by certain MMP inhibitors. More recently this proposal has been validated by detection of specific MMP degradation products in vivo (10–13). The predominant site at which MMPs cleave in the IGD is Asn 341→Phe. Cleavage at this site was first reported for MMP-3 (stromelysin-1) (14, 15). Each MMP subsequently tested, including MMP-1(16), -2 (17, 18), -7 (17), -8 (16), -9 (17, 18), -13(19), and -14 (20, 21) has been shown to exhibit the same specificity for cleavage at Asn341→Phe. Two enzymes, MMP-8 and atrolysin C, have been shown to cleave aggrecan at both the MMP and aggrecanase sites (22–24). It is now abundantly clear that both aggrecanase, which is a member of the ADAMTS family (25), and MMPs have a role in human disease (12, 13). The recent development of neoepitope antibodies (13, 26–30) enable aggrecanase and MMP activities to be distinguished from each other and compared. However, the relative contribution of aggrecanase and MMPs to aggrecan turnover has not been quantitated, nor is it clear whether one activity is more predominant in normal catabolism and the other more so in pathology. Most studies have been done with animal tissue but the relative involvement of MMPs and aggrecanase appears to differ between species (31), making interspecies comparisons difficult, and adding little to our understanding of human aggrecanolysis. In this paper we describe a production of a human rG1-G2 fragment. The rG1-G2 retains its functional properties of binding to hyaluronan and link protein and exhibits a typical double globe structure. The rG1-G2 is cleaved in vitro by aggrecanase and MMPs at the authentic Glu873→Ala and Asn341→Phe bonds, respectively, and therefore represents a valuable substrate for studying the role of these enzymes in cell-mediated aggrecanolysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—A baculovirus expression system was from CLONTECH. Grace’s insect medium, SF 9001 serum-free medium, yeastolate, and lactalbumin hydrolysate were from Life Technologies Inc. Restriction endonucleases, N-glycosidase F (EC 3.5.1.52), O-glycosidase (EC 3.2.1.97), keratanase (EC 3.2.1.103), and a chemiluminescence blotting substrate kit were from Roche Molecular Biochemicals, Germany. The ECL-plus enhanced chemiluminescence kit was from Amersham Pharmacia Biotech. AmpliCycle Sequencing Kit and Taq DNA polymerase were from Perkin-Elmer. Oligonucleotides were synthesized by Bresatec, Australia. The TNT in vitro transcription/translation kit was from Promega. Translation grade l-[35S]methionine (1000 Ci/mmol) was from NEN Life Science Products Inc. Bromoacetic acid N-hydroxysuccinimide ester was from Sigma. Recombinant human interleukin-1α (IL-1α) was from Genzyme Diagnostics. All-trans-retinoic acid was from ICN Biochemicals. Keratanase II was from Seikagaku, Japan. The Biosep-SEC S4000, 300 × 7.8-mm analytical column was from Phenomenex and the BioSil SEC-400, 600 × 21.5-mm preparative column was from Bio-Rad. The 10-mer synthetic peptides for competition assays, EDVFDPENF, GEDVFDPEN, TGEDVFDP, YTEDFDVP, LPNRITEGE, PLPRNITE, and ELPLPRNITE, were from AUSPEP, Australia. Hyaluronan-coupled-Sepharose (HA-Sepharose) was kindly provided by Professor T. Hardingham, University of Manchester, United Kingdom. The following enzymes were generously provided by Professor G. Murphy, University of East Anglia, Norwich, UK: recombinant human MMP-1 (32), recombinant human MMP-3 (33), recombinant human MMP-7 (34), MMP-9 purified from human gingival fibroblast conditioned media (35), and recombinant human MMP-10 (34). Recombinant human pro-MMP-13 (36) and recombinant human pro-MMP-8 were generously provided by Dr. V. Knapke and Professor G. Murphy, University of East Anglia, Norwich, UK. The snake venom hemorrhagic toxin HT-d (atrolysin C) was purified from rattlesnake venom (37) and kindly provided by Professor J. Fox, University of Virginia, Charlottesville, VA. The MMP inhibitor XS309 ([3S(2R*,2’R*,2”R*,2”’S*)]-hexahydro-2-R-[2-(hydroxyamino)-1-methyl-2-oxyethy]-4-methyl-1-oxopyt-yl-N-methyl-3-pyridiazinecarboxamide) (38) was synthesized at DuPont Pharmaceuticals Co. (Wilmington, DE). All other reagents were of analytical grade.

**Construction of the Human Aggrecan G1-G2 Plasmid**—A DNA clone pSA005 (4) encoding the signal peptide sequence, the G1 and G2 globular domains, and the IGD of human aggrecan (nucleotides 61–2090) was modified by polymerase chain reaction to include a polystyrene tag, a stop codon, and an EcoRI restriction site at the 3′ end. Primers Aggr1 (5′-CTCTCGCCACAGGCC-3′) and HisAgg (5′-CGAATTCTGGTATGCTGTGATCACGAAAGCC-3′) were used to amplify a 312-bp fragment which was digested with restriction endonucleases SacI and EcoRI to generate two bands, 202 and 110 bp. The 202-bp band containing the stop codon, polystyrene tag, and EcoRI site was purified and used to replace a 174-bp cassette excised from pSA005. The G1-G2 construct was subcloned into the pBacPAK8 transfer vector between the polystyrene promoter and polyadenylation signal, generating pBacPAK8-G1-G2, then sequenced using AmpliTag Cycle sequencing.

**Production of Recombinant Baculovirus**—In the initial experiments, IPLB-SF21 (SF21) cells were maintained in culture at 27°C in TFM-FH medium (Grace’s insect medium supplemented with 0.33% yeastolate and 0.33% lactalbumin hydrolysate) containing 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. In later experiments, SF21 cells were cultured in S9001 serum-free medium. Recombinant virus was produced by co-transfecting SF21 cells with pBacPAK-G1-G2 transfer vector and non-viable BacPAK6 viral DNA. Homologous sequences within the BacPAK viral expression vector, and the pBacPAK transfer vector allow double recombination to occur. The recombinant enzyme was produced by injecting recombinant G1-G2 cDNA, the polystyrene promoter, and the polyadenylation sequences to the viral ge...
nome, which renders only the recombinant virus viable. Polymerase chain reaction using the Aggr 1 and HisAgg primers to amplify a 312-bp fragment confirmed the identity of the recombinant virus. Plaque assays showed that the recombinant virus stock contained 5 × 10⁵ plaque forming units. Subsequently, SD21 cells (12 × 10⁶/175-cm² flask) were infected with the virus forming one plaque per flask in 1 h at room temperature. The medium was replaced and the cells incubated for a further 96 h. rG1-G2 secreted into the medium was detected by silver stain and immunoblotting with monoclonal antibody 1-C-6 (39).

Isolation and Purification of Recombinant Human G1-G2—Recombinant G1-G2, in the culture supernatant, was isolated by affinity chromatography using either BioSil SEC 400 or BioSep-SEC S4000 columns eluted in buffer containing 4 M GdnHCl, 50 mM sodium acetate, pH 5.8. Fractions were concentrated by SDS-PAGE on a Philips 410LS as described previously (41).

Solid Phase Binding of rG1-G2 to Matrix Ligands—The binding of rG1-G2 to link protein, G1 domain, and hyaluronan was demonstrated by incubation of [³²P]-labeled rG1-G2 with nitrocellulose membrane containing dots of purified ligands. rG1-G2 was iodinated using the chloramine-T method (19, 100 μg/ml of link protein, 4 M GdnHCl and G1 domain, both purified from pig laryngeal aggrekan, hyaluronan, bovine serum albumin, and uniduted 4 M GdnHCl were dotted onto a nitrocellulose membrane using a dot-blotting apparatus and washed through with 2 ml of PBS. The membrane was blocked with 5% skim milk/PBS for 3 h at room temperature, followed by overnight incubation with [³²P]-rG1-G2 (2 × 10⁶ cpm/ml) at 4 °C. The membrane was washed in 0.1% Tween/PBS for 45 min, then exposed to a phosphor screen for 24 h. The phosphor screen was scanned on a Storm 840 PhosphorImager (Molecular Dynamics) and the data analyzed by densitometry.

Antibodies—The neoeptope monoclonal antibodies AF-28 (29) and BC-5 (26) recognizing the N-terminal sequences F³⁴⁹FGGG and A³⁷⁴RGSV, respectively, have been described. Monoclonal antibody 1-C-6 (Developmental Studies Hybridoma Bank, University of Iowa, Dept. of Biological Sciences, Ames, IA) recognizes an epitope within the sequence SPEQLQAAYG in the G1 and G2 domains (39, 47). Monoclonal antibody 5-D-4 recognizing a highly sulfated 5 disaccharide unit of keratan sulfate (48, 49) was a gift from Professor B. Caterson, University of Wales, Cardiff, UK. Polyclonal anti-human G1 domain antiserum was a gift from Professor T. Hardingham, University of Manchester, United Kingdom. Aggreccanase-digested samples were analyzed on 8–16% Tris glycine gradient gels. BC-3 Western blots were done with goat anti-mouse IgG linked to alkaline phosphatase and the blots were developed with a Western blue phosphatase system. AF-28, anti-DIPEN, 1-C-6, and 5-D-4 Western blots were developed with alkaline phosphatase and the substrate was Sigma Fast® nitroblue tetrazolium. Anti-ITEGE, anti-Lys-ITEGE, and anti-Arg-ITEGE were the same as described previously (38, 42).

RESULTS

The construct made for expression of human G1-G2 contained the first 656 amino acids of the mature aggrecan core protein and a C-terminal polyhistidine tag. The construct was sequenced and found to be identical with the published cDNA sequence (4), except for two modest changes reported previously (50); one that changed Pro³⁶³-Gly³⁶⁴ to Leu³⁶³, Arg³⁶⁴, and insertion of CTG (coding for leucine) between nucleotides 1872 and 1873 of the published cDNA sequence. The construct was cloned into pGemZ11(f+), and analyzed by cell-free transcription and translation. The [³⁵S]methionine-labeled translation product migrated on SDS-PAGE with an apparent molecular mass of 90,000, slightly higher than the predicted size of 73,290 daltons (data not shown). The size of the translation product
product confirmed that the human rG1-G2 was full-length. rG1-G2 of the same size was also present in the medium and cells of SF21 cells infected with recombinant virus (data not shown).

**Purification of rG1-G2**—The specific and high affinity binding of the aggrecan G1 domain to HA (51) suggested that HA binding could be exploited for the purification of rG1-G2, provided the expressed protein was correctly folded. Sepharose-linked HA efficiently bound rG1-G2 under associative conditions (Fig. 2). No rG1-G2 was recovered in the column washes (Fig. 2, lanes 3 and 4) and the rG1-G2 was eluted by dissociation from HA with 4 M GdnHCl, 50 mM sodium acetate, pH 5.8 (Fig. 2, lanes 5, 6, and 9). Silver stain analysis showed that the majority of contaminating proteins present in the fetal calf serum, and other proteins secreted from the insect cells, did not bind to HA-Sepharose and were recovered in the unbound fraction (Fig. 2, lanes 2 and 8). Thus, HA-Sepharose was a highly efficient first step in purification of the rG1-G2. Subsequent size exclusion chromatography in acetate-buffered 4 M GdnHCl was used to purify the rG1-G2 to homogeneity. Fractionation of the sample on a Bio-Sep SEC 400 column showed one major peak in the included volume of the column that was purified to homogeneity by refractionation on the same column (Fig. 3). The maximum yield of rG1-G2 obtained from 240 ml of serum-free culture medium and 8 x 10^6 cells was approximately 7.7 mg.

**Rotary Shadowing Electron Microscopy of rG1-G2**—Native G1-G2 purified from pig laryngeal aggrecan (40) and human rG1-G2 were analyzed by rotary shadowing electron microscopy. Several fields of each were observed at comparable magnifications and representative structures are shown in Fig. 4. Both samples showed a similar “double-globe” structure as the predominant feature in the field. The native pig material (Fig. 4B) appeared slightly larger, with the separation between the globular domains longer. The native pig G1-G2 measured approximately 39 nm globe-to-globe, in excellent agreement with previous reports of the length of G1-G2 measured by similar means (52). The human rG1-G2 preparation (Fig. 4A) appeared less extended, with larger globular regions and the approximate length of these structures was 29 nm.

**Interaction of rG1-G2 with Matrix Ligands**—Link protein and the aggrecan G1 domain participate in aggregate formation by their noncovalent binding to HA and each other. The ability of rG1-G2 to interact with link protein, G1 domain, and HA was investigated in a solid phase binding experiment, where ligands were immobilized on a nitrocellulose membrane and allowed to interact with 125I-rG1-G2 (Fig. 5). The order for the specific binding of 125I-rG1-G2 was HA >> link protein >> G1 domain, and there was no binding of rG1-G2 to bovine serum albumin at any concentration tested. The results of these interaction studies are comparable with earlier interaction studies between isolated native aggrecan components (40, 53).

**Digestion of rG1-G2 with Deglycosylating Enzymes**—Native G1-G2 purified from pig laryngeal aggrecan carries approximately 30–40 kDa of keratan sulfate (17) and some N- and O-linked oligosaccharides (53). To compare the glycosylation of rG1-G2 with native G1-G2, both were digested with N-glycosidase F, O-glycosidase, keratanase, and keratanase II and analyzed by SDS-PAGE. N-Glycosidase F treatment of native G1-G2 decreased its average molecular size by 10–15 kDa (Fig. 6a, lane 5). The rG1-G2 was also decreased in size by N-glycosidase F treatment, but only by 2–5 kDa (Fig. 6a, lane 2). The size of native G1-G2 was unchanged by treatment with O-glycosidase (Fig. 6a, lane 6), however, O-glycosidase treatment reduced rG1-G2 by 2–5 kDa (Fig. 6a, lane 3). This data shows that rG1-G2 carries small amounts of N- and O-linked oligosaccharide. It shows that short O-linked disaccharides with the structure Galβ1-3GalNAc are present on rG1-G2, since longer structures are not hydrolyzed by the O-glycosidase used in this study. Native G1-G2 most likely contains more complex oligosaccharide structures, since it was resistant to digestion by O-glycosidase. rG1-G2 does not carry keratan sulfate chains since keratanase and keratanase II digestion failed to alter the size of the rG1-G2 (Fig. 6b, lanes 2 and 3) but decreased the size of native pig G1-G2 by approximately 30 kDa (Fig. 6b, lanes 5 and 6). Enzyme-linked immunosorbent
assay of G1-G2 with monoclonal antibody 5-D-4 also failed to detect the highly sulfated pentameric structures typically found in keratan sulfate, and highly expressed on native human and pig aggrecan (results not shown).

**Digestion of rG1-G2 with Aggrecanase—** If rG1-G2 is to be a suitable *in vitro* substrate for investigating aggrecan cleavage by purified proteinases or cultured cells, it must show the same cleavage site specificity for aggrecanase (Glu373 Phe) as the native molecule. Aggrecanase activity was generated in conditioned media from IL-1-stimulated bovine nasal cartilage (42). rG1-G2 was digested with aggrecanase in the presence of a potent MMP inhibitor ZS309, and the products detected by Western blotting with monoclonal antibody BC-3 specific for the A374PGSV N-terminal sequence and polyclonal anti-ITEGE specific for the C-terminal sequence (Fig. 1). A single bond of M, 45,000 was detected with BC-3 (Fig. 7a) and a single G1 species of M, 56,000 was detected with anti-ITEGE (Fig. 7b), confirming that aggrecanase cleaved the rG1-G2 at the Glu373 Ala bond. No bands were detected with AF-28 or anti-DIPEN antibodies (results not shown). Competitive enzyme-linked immunosorbent assay experiments confirmed the specificity of the anti-DIPEN antiserum since the 10-mer peptide with an extension of 1 amino acid (EDFVDIPENF) was almost 2 orders of magnitude less competitive than the true neoepitope peptide (PLPRNITEGE). 10-Mer peptides truncated by one (LPLPRNITEG) or two (ELPLPRNITE) amino acids gave no competition at all (results not shown).

**Digestion of rG1-G2 with MMPs—** Recombinant human MMP-13 (36) was used to digest rG1-G2 since this MMP is relatively abundant in cartilage (54–56) and the products of native pig G1-G2 digestion by MMP-13 have been characterized previously (19). Following MMP-13 digestion, three rG1-G2 fragments were detected by silver staining (Fig. 8a, lane 2). The fastest migrating band with M, 44,000 (Fig. 8a, fragment 3) was shown by Western blotting with anti-human G1 antiserum to be the G1 domain (Fig. 8b, lane 2). Based on the results of MMP-13 digestion of native pig G1-G2 (19) we predicted that MMP-13 digestion of the human rG1-G2 would yield two G2 bands by cleaving at both the major and minor MMP sites. A G2 product of M, 55,000 with F342FGVG N terminus was detected with monoclonal antibody AF-28 (Fig. 8c, lane 2), and corresponded in size to fragment 1 in the silver stain gels. Fragment 2 was not analyzed further, however, it is likely that it is the smaller G2 fragment resulting from hydrolysis of the Pro384 Val bond in the IGD (19). These results indicate that MMP-13 has the same specificity for human rG1-G2 as it does for native pig G1-G2. No bands were detected with BC-3 or anti-ITEGE antibodies (Fig. 1), confirming previous work (19) that showed MMP-13 does not have aggrecanase activity (results not shown).

Five additional MMPs as well as two non-MMP proteinases were incubated with the rG1-G2 substrate and the products analyzed by SDS-PAGE and Western blotting (Fig. 9). MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-9 (gelatinase B), and MMP-10 (stromelysin-2) all cleaved rG1-G2 at the Glu373 Phe bond second. rG1-G2 was also cleaved sequentially by MMP-8. Incubation of native pig G1-G2 or bovine aggrecan (23) with MMP-8 showed that the enzyme cleaved its substrates in a sequential manner, hydrolyzing the Asn341 Ala bond second. rG1-G2 was also cleaved sequentially by MMP-8. Digestion of rG1-G2 with increasing concentrations of MMP-8 showed that the AF-28 epitope was present at all concentrations, but was decreased at the higher concentrations when
BC-3 epitope began to appear. DIPEN\textsuperscript{341} epitope in contrast remained constant and did not decrease (data not shown). The snake venom proteinase atrolysin C cleaves bovine aggrecan at the Asn\textsuperscript{341}\textsuperscript{2}Phe and Glu\textsuperscript{373}\textsuperscript{2}Ala sites, but in an independent rather than sequential manner (24). When rG1-G2 and native pig G1-G2 were digested with atrolysin C at 0.1 M (data not shown) and 1.0 M, digestion products were detected by silver stain (Fig. 10\textsuperscript{a}) and ITEGE\textsuperscript{373} immunoreactivity (Fig. 10\textsuperscript{b}). AF-28 fragments were detected for native G1-G2 (Fig. 10\textsuperscript{c}, lane 4) but no AF-28 fragments were found in atrolysin C digests of rG1-G2 (Fig. 10\textsuperscript{c}, lane 2), indicating that atrolysin C was unable to cleave rG1-G2 at the MMP site. To determine whether the absence of keratan sulfate chains on rG1-G2 conferred resistance to atrolysin C cleavage at the MMP site, we analyzed the susceptibility of native G1-G2 to atrolysin C following removal of keratan sulfate chains by keratanase (Fig. 10, d-f). We found that native G1-G2 was readily cleaved at the MMP site, irrespective of whether or not keratan sulfate chains had been enzymatically removed (Fig. 10, e and f). Anti-ITEGE\textsuperscript{373} immunoreactive products were also present following keratanase digestion of native G1-G2 (data not shown). These results suggest that the presence or absence of keratan sulfate chains per se does not affect the specificity of action of atrolysin C.

Incubation of rG1-G2 with Conditioned Explant Culture Medium—Studies by Hughes et al. (31, 57) have shown that aggrecanase present in chondrocyte-conditioned medium can be monitored using a chimeric recombinant substrate. We have tested whether aggregcanase present in conditioned media from cartilage explant cultures can be monitored with rG1-G2. Western blotting with anti-ITEGE (Fig. 1) rabbit sera revealed the presence of aggregcanase-derived G1 fragments following incubation of rG1-G2 with media from IL-1\alpha or retinoate-stimulated cultures (Fig. 11, lanes 2 and 3). No aggregcanase-derived ITEGE fragments were found in samples incubated with media...
from control cultures (Fig. 11, lane 1), nor was anti-ITEGE signal present in lanes containing conditioned medium alone (data not shown). No AF-28 or DIPEN fragments (Fig. 1) were found in any samples, suggesting that there were no active MMPs present in conditioned media from control, IL-1α, or retinoic acid-treated cultures (data not shown). These data demonstrate the utility of rG1-G2 for the study of cell-mediated aggrecanolyis.

**DISCUSSION**

**Properties of rG1-G2**—In this paper we report the utility of rG1-G2 for the study of aggrecanolyis in *vitro*, by demonstrating that the intrinsic properties of MMP and aggrecanase cleavage site specificity, as well as HA, link protein, and G1 binding, are retained. MMP digestion of rG1-G2 yields G1 and G2 products of M, 44,000 and 55,000, respectively, while aggrecanase digestion yields G1 and G2 products of M, 56,000 and 45,000, respectively. These products are readily identified with neoepitope antibodies in *vitro*. Recombinant fusion proteins comprising various domains of aggrecan G1-G2 have been reported previously. An artificial aggrecan substrate (rAgg1) comprising the IGD of human aggrecan flanked by the marker sequences FLAG™ at the N terminus and the human immunoglobulin G1 constant region at the C terminus has been expressed in COS cells (57) and shown to be processed by aggrecanase and MMPs (31). A G1-G2 fusion protein expressed in an embryonal kidney cell line, fused with the laminin γ1 chain at the C terminus and an interleukin-2 signal peptide and FLAG™ sequence at the N terminus has also been produced and shown to bind hyaluronan (58). This paper describes for the first time the production of a natural rG1-G2 that has been expressed in insect cells, giving significantly greater yields of recombinant protein compared with mammalian expression, but without full glycosylation.

The rG1-G2 has been produced in high yield and purified by HA-affinity chromatography. We observed that the SF21 cells maintained in serum-free medium produced rG1-G2 that migrated as a broader band on gels than the rG1-G2 secreted by cells maintained in fetal calf serum. The broader band presumably reflects a greater degree of heterogeneity, which may result from more extensive glycosylation and differential processing by the signal peptidase (59, 60). During early experiments to develop and test methods for purification we found that freezing unpurified rG1-G2 in the conditioned culture medium reduced the final yield of purified protein, but that purified rG1-G2 was stable to freeze-thawing. We also found that HA-Sepharose affinity chromatography was far superior compared with metal affinity chromatography utilizing the polyhistidine tag.

Measurements by rotary shadowing electron microscopy (52) for the length of the G1-IGD-G2 region (double-globe) have been derived for aggrecan purified from bovine nasal cartilage, pig laryngeal cartilage, and the swarm rat chondrosarcoma. The native pig G1-G2 analyzed in the present work measured approximately 39 nm globe-to-globe, in excellent agreement with previous reports (52, 61). The enzymatic removal from bovine aggrecan of chondroitin sulfate chains caused a shortening of the distance between the G2 and G3 domains from 405 ± 37 to 263 ± 27 nm, which is approximately 35% (61). The authors suggested that charge repulsion or steric effects of chondroitin sulfate chains were responsible for the extended length of the E2 domain between G2 and G3. In the present work, the human rG1-G2 measured 29 nm, which is approximately 29% shorter than native G1-G2. One possible explanation is that in rG1-G2, the lack of keratan sulfate substitution in the IGD contributes to the shortened length of the E1 domain. However, detailed analyses of double globe structures from rat chondrosarcoma, bovine nasal, and pig laryngeal cartilage have shown that the length of the E1 segment between G1 and G2 is remarkably constant, at about 25 nm (52). Since there is no evidence for keratan sulfate substitution on rat chondrosarcoma aggrecan, this would suggest that keratan sulfate is not a key factor in determining the length of the E1 domain, and that perhaps an altered pattern of O- or N-linked glycosylation may contribute to shortening of E1 in rG1-G2.

**rG1-G2 Glycosylation**—The expression of keratan sulfate-containing proteins by insect cells has not been reported. In our hands, there was no substitution of keratan sulfate on rG1-G2, suggesting that SF21 cells lack the specialized glycosylation machinery necessary for keratan sulfate biosynthesis. Similarly, the chondroitin/dermatan sulfate proteoglycan, decorin, was not substituted with glycosaminoglycan chains by SF21 cells (62). N-Glycosidase F releases asparagine-linked glycan chains from mammalian glycoproteins including N-linked keratan sulfate. Since keratanase treatment reduced the size of the native G1-G2 by about 30 kDa and N-glycosidase F by only about 15 kDa, it appears that about 50% of the keratan sulfate on native pig G1-G2 is N-linked to the protein core. Whether this N-linked keratan sulfate is present on the same asparagine residues suggested by Barry et al. (63) for bovine aggrecan HABR is under investigation.

N-Linked glycosylation is common in insect cells and a small amount was present on rG1-G2, albeit at 50–85% less than that on native G1-G2. The N-linked glycans present on rG1-G2 are likely to comprise mannose mannose type (Man₆₋₉GlcNAc₂) or short truncated structures (Man₆₋₉GlcNAc₂) since these are most commonly observed on proteins expressed in cells derived from S. frugiperda (64–70) and are released by treatment with N-glycosidase F. However, we cannot exclude the possibility that the N-glycans may be of the complex type with or without terminal sialic acid residues, which are uncommonly reported for these cells (66, 71). Some studies have shown that the N-linked glycans on recombinant proteins secreted from insect cells are important for functional properties. For example, ligand binding of the thromboxane A₂ receptor (72), secretion of decorin (62), and the ability of human plasminogen to be activated by urokinase (73) are all influenced by N-glycan substitution. At present we do not know whether differences in N-linked glycosylation may contribute to the inability of atrolysin C to cleave the Asn⁴¹₁ Phe bond in rG1-G2.

Studies by Arner's laboratory² have suggested that keratan sulfate substitution on aggrecan may facilitate aggrecanase activity. Treatment of bovine nasal cartilage aggrecan with keratanases I and II generated a product which was not cleaved by crude aggrecanase, whereas treatment with chondroitinase ABC did not markedly alter the substrate cleavage. However, the present data clearly show that keratan sulfate

² M. A. Pratta, M. D. Tortorella, and E. C. Arner, unpublished results.
chains are not strictly required for aggrecanase activity; this is in agreement with the demonstrated aggrecanase-mediated cleavage of other aggrecan substrates, such as rat chondroarcoma aggrecan (27) and recombinant IGd, rAgg (57) which are devoid of keratan sulfate. With respect to MMP cleavage, rG1-G2 was a much better substrate than glycosylated G1-G2 since lower enzyme concentrations and shorter digestion times were required for the maximum yield of products. The role of keratan sulfate in the control of aggrecanolyis is an emerging theme and future studies with human rG1-G2 expressed in a system able to elaborate keratan sulfate will allow us to address this issue directly.

The susceptibility of rG1-G2 to atrolysin C was of interest because this represols is able to cleave bovine aggrecan at both the MMP and aggrecanase sites (24). rG1-G2 was not cleaved at the MMP site by atrolysin C even though it was efficiently cleaved at the aggrecanase site. When we investigated whether native glycosylated G1-G2 was cleaved at the MMP site by atrolysin C we found not only that it was, but that native G1-G2 digested with keratanase was also cleaved at the MMP site. This result suggests that keratan sulfate substitution in the IGD has no influence on the specificity of atrolysin C for cleavage at the MMP site, and that factors other than lack of keratan sulfate chains on rG1-G2 abolishes atrolysin C cleavage at Asn441 ▼ Phe. Using circular dichroism and fluorescence spectroscopy, Krishnan et al. (74) have observed that the conformation of biglycan is variably influenced by whether it is synthesized with or without a glycosaminoglycan chain, and that removal of the chain after secretion has no appreciable influence on its conformation. Although biglycan and aggrecan core proteins are not at all similar and the extent of glycosylation on each is vastly different, it is possible to speculate that the addition of keratan sulfate chains to the nascent aggrecan core protein during biosynthesis may result in a structural conformation that is permissive for atrolysin C cleavage at the Asn441 ▼ Phe site. In such a scenario, subsequent removal of keratan sulfate would not alter protein conformation or abolish atrolysin C cleavage at Asn441 ▼ Phe. However, rG1-G2 synthesized without keratan sulfate chains would possibly adopt a different conformational structure that allows binding of the enzyme (since the substrate is cleaved at the Glu373 ▼ Ala bond), but is inhibitory for cleavage at the Asn441 ▼ Phe site.

In Vitro Aggrecanysis—Soluble active aggrecanase has been generated in conditioned media from interleukin-1-stimulated bovine nasal cartilage (42), and we now show that it can also be harvested from porcine articular cartilage following stimulation with IL-1α or retinoic acid. We show that in contrast to cell culture (31), conditioned medium from explant cultures does not contain active MMPs, since DIPEN434 and F424FGVG neoepitopes (Fig. 1) were not detected in digests of rG1-G2 with either control or stimulated culture medium. There are several possible reasons why active MMPs are not released in cartilage cultures, but are released in chondrocyte cultures, and why active aggrecanase is released. One is that there are significant levels of TIMPs present in the cartilage matrix. MMP cleavage in tissue slices most likely occurs tran- siently, prior to inhibition by TIMPs. The IC50 for inhibition of aggrecanase by TIMP-1 is 210 nM, whereas the IC50 for inhibition of MMPs by TIMP-1 is about 100-fold lower (42). Furthermore, aggrecanase is not inhibited by TIMP-2. Thus, MMPs in tissue are much less likely to escape inhibition by TIMP than aggrecanase. Second, MMPs bind to extracellular matrix, and indeed there are studies to suggest that active stromelysin may be trapped in the cartilage matrix through tight binding, perhaps to its natural substrate (75). At present there is no infor-
