Cleavage of the Carboxyl Tail from the G3 Domain of Aggrecan but Not Versican and Identification of the Amino Acids Involved in the Degradation*

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Aggrecan, a major structural proteoglycan in cartilage, contains three globular domains, G1, G2, and G3, as well as sequences for glycosaminoglycan modification. A large number of proteases are implicated in aggrecan cleavage in normal metabolism, aging, and arthritis. These proteases are known to cleave at the IGD, KS, and CS domains. Here we report for the first time evidence of cleavage at a novel site, the carboxyl tail of aggrecan. Results from deletion mutants of the tail indicated that the likely cleavage sites were two consensus sequences, RRLXXK and RSPR, present in the aggrecan analogs of many species. This was confirmed by site-directed mutagenesis. A construct containing two G3 domains (G3G3) was also found to cleave between the G3 duplicates. When G3 tail was linked to a glycosaminoglycan-modifying sequence, it was protected from cleavage. Furin inhibitor also reduced the levels of tail cleavage. The carboxyl tails of chicken and human versican were not cleaved, despite the presence of the consensus sequence. Our studies indicate that the basic amino acids present in the tail play an important role in cleavage, and this mechanism is specific to aggrecan.

Members of the family of large aggregating chondroitin sulfate proteoglycans include aggrecan, versican, neurocan, and brevican. All are composed of a signal peptide, a globular domain named G1, a large fragment for chondroitin sulfate modification (CS sequence), and a globular domain G3 (1). The G1 domain, which is homologous to link protein, contains one immunoglobulin (IgG)-like domain and two tandem repeat motifs (2–4). Aggrecan is unique in containing an additional globular domain G2 that is structurally similar to the tandem repeats of the G1 domain. An inter-globular domain (IGD) is located between the G1 and G2 domains, and a segment modified by keratan sulfate (KS sequence) is situated between the G2 domain and the CS sequence. The G3 domain is composed of one or two alternatively spliced epidermal growth factor (EGF)-like motif(s), one lectin (also called carbohydrate recognition domain or CRD)-like motif, one complement binding protein (CBP)-like motif, and a short C-terminal tail (2, 5, 6). The structures of these motifs, especially the tail, are highly conserved in aggrecan across species boundaries.

In the early stages of cartilage development, versican is the predominant proteoglycan and is believed to play an important role in tissue development. After cartilage maturation, versican is replaced by aggrecan, which facilitates the formation of a matrix network for resilience and load-bearing. Aggrecan processing seems to be regulated, in part, by the G3 domain. This was initially observed in the chicken disorder nanomelia, which produces a lethal phenotype in homozgyous form (due to failure of chondrogenesis and osteogenesis) and dwarfism in heterozygote form (7). Cartilage of the nanomelic mutant lacks aggrecan in its matrix. It was discovered that the core protein of this mutant aggrecan is truncated as a result of a premature stop codon at the N-terminal side of G3 (2). In addition, its secretion is hindered and no modification by glycosaminoglycan (GAG) chains occurs (7). Recently, it was reported that the G3 and G1 domains play roles in processing of recombinant aggrecan and versican (8–14).

Extensive aggrecan degradation occurs during normal cartilage metabolism, aging, and joint diseases (15, 16). In mature cartilage, up to half of the aggrecan population loses its G3 domain. The other domains are also subjected to cleavage, resulting in the release of degraded fragments in the matrix. The exception is the G1 domain, which is trapped and retained by hyaluronan. Loss of aggrecan is a major feature of cartilage degradation associated with arthritis (6). In the early stages of rheumatoid arthritis, the chondroitin sulfate-bearing sequence of aggrecan, the CS domain, is preferentially released. The synovial fluid of patients with osteoarthritis and joint injury contains fragments of degraded aggrecan (17). A large number of proteases are known to cleave aggrecan, and their target sites are located in the IGD, KS, and CS domains (15, 18–21). No protease has yet been reported to cleave inside the globular domains, or in the carboxyl tail.

Our preliminary studies suggested that the carboxyl tail of aggrecan is removed during product secretion. After transfecting COS-7 cells with a His-tagged (at the C terminus) G3...
construct, we did not detect the His-tagged G3 product in culture medium but detected this product in cell lysate. An antibody against another epitope at the amino region allowed detection of product in both culture medium and cell lysate. Hence, we hypothesized that the G3 tail was removed after product secretion. The present study was designed to determine the mechanism of this process, to identify amino acid motifs that may be involved in this degradation, and to determine whether this process is specific to aggrecan or common to the proteoglycan family.

**EXPERIMENTAL PROCEDURES**

**Materials—**PCR amplification kit, Taq DNA polymerase, and restriction endonucleases were from Roche Molecular Biochemicals and New England Biolabs. DNA marker was from Fermentas MB. Bacterial growth medium was from Difco. Prestained protein marker was from New England BioLabs. Lipofectin, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Hanks’ balanced salt solution, trypsin/EDTA, and T4 DNA ligase were from Invitrogen. ECL Western blot detection kit was from Amersham Biosciences, Inc. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Sigma Chemical Co. Anti-His-tag monoclonal antibody and DNA Midi-prep kit were from Qiagen Inc. Tissue culture plates (6-well, 12-well, and 100 mm) were from Nunc Inc. Protease inhibitors Amastatin and E64 were from Calbiochem. A furin inhibitor (decanoyl-RVKR-chloromethylketone) was from Bachem (King of Prussia, PA). All other chemicals were from Sigma. COS-7 cells were from American Type Culture Collection. The cells were cultured in DMEM supplemented with 5% FBS at 37 °C in a humidified incubator containing 5% CO2.

**Strategy for Generation of Recombinant Constructs—**In this study, a total of 43 recombinant constructs was used: G3His, HisG3, C2 (C representing the last cysteine residue of aggrecan), C7, C13, C16, C20, C23tail, C7tail, C13tail, C16tail, Muttail, CR8P, CR9P, CK12A, CR13S, del8–12, del8–12R13S, del8–12R13S, del8–12R13S, CR16P, G3KRTS, G3allmut, HuC8–13, HuC13–16, HuC16–19, G3KS, G3GS, C2GS, C7GS, C13GS, C9FG3, CK12AG3, CR13SG3, G3RTSOG3, G3almutG3, del8–12G3, huCR–13G3, hvG3A, hvG3C, cvRSFRA3G3, hvRSFRA3G3, and cvG3ΔEGFPHis. G3His, G3KS, and cvG3ΔEGFPHis have been described in our previous publications (10, 22–25; for structures see Figs. 1, 6, and 9 of this paper). The G3His construct was generated by ligating together the leading peptide (LP) of link protein, aggrecan G3 domain and a His-tag. The leading peptide contained link protein signal peptide and an epitope recognized by the monoclonal antibody 4B6 (26). The G3KS construct contained the leading peptide, aggrecan G3 domain, aggrecan KS domain, and a His-tag.

The G3His construct was generated by ligating two fragments, the leading peptide containing a His-tag and the G3 domain, into pcDNA3. The leading peptide containing a His-tag was produced using two primers, LP40CHis/XhoI and LPNPKazak/EcoRI (for sequences see Table 1), in a PCR reaction and used as a template for a PCR reaction using two primers, CSDF/I 2” and CS/–2” to produce a PCR product of 982 bp. The amplified fragment was then digested with EcoRI and XhoI, purified, and ligated with the G3 fragment, which was derived by digesting the G3His construct, into pcDNA3.

The remaining 39 constructs were based on the G3His construct. They all contain the leading peptide, the CRD and CBP motifs, a modified tail, and the His-tag. Modification of the tail was performed through either systematic incremental deletion, site-directed mutagenesis, or engineering of a potential site for cleavage from other species or proteoglycans into the tail.

Ten constructs were made to contain incremental deletion of the tail. Two types of deletion were made. In the first type, the entire tail was incrementally deleted producing 5 constructs (C2, C7, C13, C16, and C20). This type was based on the plasmid Type I (see Fig. 2A). The end of the tail contains a restriction enzyme site XhoI (encoding two amino acids of CRD) followed by a His-tag. The original XhoI site on the vector was inactivated by ligation with the restriction enzyme site NheI. The primers NheIHisXhoI&XhoI and CRDNXhoI were used in a PCR with aggrecan G3 domain as a template. The product was purified, digested with XhoI and NheI, and inserted into XhoI-digested G3His construct to generate the Type I construct. To generate incremental deletion mutations, two primers were used in each PCR: one complementary to the 5’ terminus of G3 domain (CRDNXhoI) whereas the other was complementary to the desired 3’ region of the tail. For example, primer C2CXhoI and CRDNSXhoI were used in a PCR using G3 as template. The PCR product was purified, doubly digested with XhoI and XbaI, and ligated with XhoI- and XbaI-digested Type I plasmid to produce construct C2. Primer C7CXhoI was combined with CRDNXhoI to produce the construct C7. C13CXhoI was used for production of construct C13, whereas C16CXhoI was used for construct C16 and C20CXhoI was used for construct C20 (see Fig. 2B). To produce the second type of incremental deletion, nine amino acids motifs from the extreme C-terminal end of the tail were linked to the His-tag in a PCR, using the primers XbaItailHis and pcDNA3XmaI (complementary to nucleotides 2091–2096 of the vector). The PCR product was digested with XbaI and XmaI and inserted into XhoI- and XmaI-digested construct C2 resulting in the construct C2tail. Replacement of the XhoI-XmaI fragment from the construct C7 with the PCR product produced construct C7tail. Constructs C13tail, C16tail, and C20tail were generated in the same way.

Another ten constructs contained site-directed mutations in the tail that could be divided into three groups. In Group 1, the first potential consensus sequence (amino acids RRLYKR, see Fig. 3A) was mutated: in each construct, one basic amino acid was mutated to a non-basic amino acid. For example, two primers, CSDFBamHI and CR8PbamHI were used in a PCR with C13tail as a template. The primer CR8PbamHI generated a single mutation at amino acid C9 (R → P) and produced the construct CR9P. Likewise, combined with CSDFBamHI, primers CK12AbamHI and CR13SbamHI generated the construct CR13Sw. In the same way, in the second group, we linked the second consensus sequence to construct C2tail (see Fig. 3A). This was performed by using two primers, CSDFBamHI and del8–12, which deleted the first motif, amino acids 8–12, in a PCR with G3 domain as a template. The PCR product was purified, digested with XhoI and XbaI, and ligated into XhoI- and XbaI-digested C2test. Combined with primer CSDFBamHI, primer delR13SbamHI was used to generate a mutation at amino acid 13 to produce the construct delR13S, and primer delR16PbamHI was used to generate a mutation at amino acid 16 to produce the construct delR16P.

In the third group, each construct contained multiple mutations. Construct Muttail was generated in a PCR using two primers, CSDFBamHI and muttailXhoI. The PCR product was inserted into XhoI- and XbaI-digested C2test. Construct G3KRTS was generated using two primers, CSDFBamHI and G3KRTSXhoI, and, after similar treatment, the PCR product was inserted into XhoI- and XbaI-digested plasmid C20tail. Using a similar method, the primers G3allmutXhoI and CSDFBamHI were used to produce the construct G3allmut (see Fig. 4A).

Four constructs contained consensus sequences obtained from bovine, human, dog, mouse, or rat aggrecan (see Fig. 5). These constructs contained a structure: the putative cleavage motif was engineered into the construct C7tail. For example, the sequence RLRKQR from the first potential cleavage site of rat, human, or dog was linked to 3’ of the construct C2 by using two primers, CSDFBamHI and HuC8–13XhoI, in a PCR. After treatment similar to that described above, the PCR product was inserted into XhoI- and XbaI-digested plasmid C7tail to produce the chimeric construct HuC8–13. The second potential protease site, RSSR, from rat, human, or dog was linked to 3’ of the construct C7 using primers HuC13–16XhoI and CSDFBamHI to produce the chimeric construct HuC13–16. The third potential protease site in human aggrecan, RHRP, was linked to 3’ of the construct C7 using primer HuC16–19XhoI and CSDFBamHI to generate the chimeric construct HuC16–19.

Fifteen constructs are based on a G3-G3 duplex. The G3G3 construct was generated by replacing the His-tag in the G3His construct with a G3His fragment (see Fig. 6). Two separate aliquots of the G3His construct were doubly digested with (i) EcoRI and SulI and (ii) XhoI and EcoRI to purify LP60G3 and G3His+pcDNA3, respectively. The two fragments were then ligated together. Ten constructs (C2G3, C7G3, C13G3, C9FG3, CK12AG3, CR13SG3, G3KRTSOG3, G3almutG3, del8–12G3, huCR–13G3) contained the mutated or truncated G3 in addition to normal aggrecan G3 (see Fig. 8). This was achieved by replacing the junction of the mutated or truncated G3 and pcDNA3 vector (XhoI-ApoI) with XhoI-ApoI-containing normal G3. The latter G3 was then digested in a PCR using aG3N3XhoI and CRDNSXhoI primers. The cvG3aG3 and hvG3aG3 were produced by linking the chicken versican G3 domain or human versican G3 domain with the normal aggrecan G3 domain as above (see Fig. 9). Generation of chicken versican G3 domain has been described by us previously (27, 28). We used an reverse transcriptase-PCR (with hvG3N3XhoI and hvG3C3XhoI as primers and glioma total RNA as template) to produce human versican.
Finally, we engineered the consensus sequence RSRR in the tail of chicken and human versican G3 domains (in constructs cvG3aG3 and hvG3aG3, respectively) using primers cvG3aCRSRRXbaI and hvG3aCRSRRXbaI in PCR reactions.

**DNA Manipulation and Clone Selection**—DNA was amplified in PCRs using pairs of appropriate primers. The reaction mixture (total volume, 100 μl) contained 200 μM dNTPs, 0.2 μg of each primer, 50 ng of template DNA, 5 units of Taq DNA polymerase, and the Mg2+-containing buffer. The reactions were carried out at 94 °C for 5 min for one cycle; 94 °C (60 s), 55 °C (60 s), and 72 °C (60 s) for 25 cycles; and a final extension at 72 °C for 10 min. DNA products were purified then doubly digested with two appropriate restriction endonucleases. The DNA was ligated into the linearized plasmid pcDNA3. The ligation reaction was carried out at 16 °C overnight. The ligation mixture was used for transformation of competent Escherichia coli strain DH5α. After clone selection, all new constructs were confirmed by DNA sequencing, performed by the Core Molecular Biology Laboratory at York University (Toronto, Ontario). The results were then compared with the published sequence.

**Construct Expression and Product Analysis**—To analyze gene expression, COS-7 cells or bovine chondrocytes were transfected transiently with recombinant constructs using Lipofectin according to the manufacturer’s instructions (Invitrogen). Briefly, the cultured COS-7 cells or bovine chondrocytes were seeded onto 12-well tissue culture plates (1.5 × 10⁶ cells/well). The cells were allowed to attach and grow overnight in DMEM containing 5% FBS. The cells were transfected once they reached 70% confluence. Lipofectin (2 μl) was incubated with plasmid DNA (2 μg) in 100 μl of DMEM followed by the addition of 900 μl of DMEM. Concurrently, COS-7 cultures were washed with 2 ml of DMEM. The Lipofectin-DNA mixture was added to the cultures followed by incubation at 37 °C for 5 h in a humidified incubator. The DNA-Lipofectin mixture was replaced with 1 ml of DMEM containing 5% FBS. Three days later, culture medium was harvested, and dead and floating cells were removed by centrifugation. Cell lysate in each well was obtained by using 200 μl of lysis buffer. Because the volume of medium in each well is 5-fold the volume of lysis buffer used, the percentage of the portions of cell lysate used in each loading well represented 5-fold more than that of the medium.

**TABLE I**

| primer       | sequence                           | restriction site |
|--------------|------------------------------------|-----------------|
| LPSKozak     | 5′_agaattgcgctgcctggccgaatg         | EcoRI           |
| LPS4CHis     | 5′_aattagctgggagctggttggtg          | XhoI            |
| NheHis       | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| CRDN         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| C2C          | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| C7C          | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| C13C         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| C16C         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| C20C         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| XbaTailHis   | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| pcDNA3       | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| HaC8-13      | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| BoC8-13      | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| HaC13-16     | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| HaC16-19     | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| delR13S      | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| delR16P      | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| CSDF         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| MutAl        | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| G3KRTS       | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| Allnt        | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| CR8P         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| CR9P         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| CR13S        | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| de18-12      | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| AG3N         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| AG3C         | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| HV3G3C       | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| hvG3N        | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| cvG3CRSRR    | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
| hvG3CRSRR    | 5′_agaattgcgctgcctggccgaatg         | XhoI            |
NaCl, 0.05% Tween 20) containing 10% nonfat dry milk powder (TBSTM) for 30 min at room temperature, and then incubated at 4 °C overnight with the monoclonal antibody 4B6 or the anti-His-tag monoclonal antibody in TBSTM. The membranes were washed with TBST (3 × 30-min washes) and then incubated for 2 h with HRP-conjugated goat anti-mouse IgG antibody (1:50,000 dilution) in TBSTM. After washing as above, the bound antibody were visualized with chemiluminescence (ECL kit, Amersham Biosciences, Inc.).

To compare the G3 tail cleavage, it was necessary to load an equal amount of secreted products to each well on Western blot. To do so, the products of each construct, as well as one control (product of C2tail construct) were pre-analyzed on Western blot probed with 4B6, which is much more sensitive than the anti-His antibody. Equal amounts of gene products, obtained by dilution, were then analyzed for G3 tail cleavage probed with anti-His antibody. Because the affinity of anti-His antibody is much lower than the 4B6 antibody, we had to use larger amounts of products (~3-fold) for anti-His staining. A reduction in the intensity of bands probed with anti-His antibody would suggest G3 tail cleavage.

**Effect of Furin Inhibitor on Product Cleavage**—COS-7 cells were transiently transfected with G3G3 construct. After overnight incuba-
tion, culture medium was replaced with fresh medium containing 5% FBS and furin inhibitor decanoyl-RVKR-chloromethylketone. Protease inhibitors Amastatin and E64 were used as controls. Cell lysate and culture medium were harvested and analyzed on Western blot as above.

**Detection Analysis**—Relative protein concentration was estimated by using a densitometer (Molecular Dynamics) according to the manufacturer’s instructions for scanning the densities of the signals on the films after Western blot. The relative intensity of each band after Western blot development is shown below the blot. In the case of product cleavage in duplex constructs, both protein bands (undegraded and degraded) were scanned and density was calculated using the following formula: degradation (%) = OD of undegraded band/OD of degraded band = OD of undegraded band.

**RESULTS**

**Cleavage of G3 Tail**—To assess the possibility of cleavage of the C-terminal tail of the chicken aggrecan G3 domain, we initially generated a G3 construct that contains the leading peptide of link protein at the N terminus. This leading peptide harbors the signal peptide and an epitope recognized by the monoclonal antibody 4B6 (26). At the C-terminal end of G3, a His-tag recognized by anti-His monoclonal antibody was added (G3His, Fig. 1A). After transfection of COS-7 cells with this G3 construct, the 4B6 antibody detected products (~48 kDa) in both culture medium and cell lysate on Western blot (Fig. 1B).

However, the anti-His antibody failed to detect the product in culture medium, but could detect the product (~48 kDa) in cell lysate (Fig. 1C). This suggested that the G3 tail has been removed after product secretion. To test this, we generated another construct (HisG3) containing both the 4B6 epitope and the His-tag at the N-terminal region of the construct. Analysis of cell lysate and culture media from COS-7 cells transfected with HisG3 on Western blot indicated that products of HisG3 (~48 kDa) were detected by 4B6 (Fig. 1D) and anti-His antibody (Fig. 1E) in cell lysate and culture medium.

**Identification of the Amino Acids Involved in the Cleavage of G3 Tail**—To investigate how the G3 tail was cleaved, ten incremental deletion constructs (C2, C7, C13, C16, C20, C2tail, C7tail, C13tail, C16tail, and C20tail) were made based on two types of parental constructs, Type I and Type II (Fig. 2, A and B). In addition, the last nine amino acids were completely mutated resulting in the construct muttail. Because all the mutations and deletions were made between two restriction sites, EcoRV on G3 domain and ApaI on the extreme 3′ cloning site, the EcoRV-ApaI fragment of the above 12 cDNA constructs (including G3His as a control) was examined using polyacrylamide gel electrophoresis to ensure that deletion had been made (data not shown). Culture medium and cell lysate from COS-7 cells transiently transfected with these 12 constructs were analyzed on Western blot probed with 4B6. All constructs were equally well expressed in both the lysate (L) and medium (M) (Fig. 2C). Probed with anti-His antibody, no significant difference in expression levels could be seen in cell lysate. However, little product was detected in the medium from G3His-, C13-, C16-, C20-, C2tail-, C7tail-, C13tail-, C16tail-, and muttail-transfected COS-7 cells probed with the anti-His antibody (Fig. 2D) suggesting that cleavage had occurred in all of these constructs. These results indicated that the sequence RLYKR (C13) was the minimum for sufficient cleavage and that this sequence was also recognized in the extended sequences RRLYKRSRPR (C16) and RRLYKRSRPSRLR (C20). On the other hand, the sequence SRPGVVHRPTH (C2tail) was not sufficient for cleavage, although its presence did not interfere with cleavage within the adjacent active motif RRLYKR (see C13 tail).

It has long been known that the amino acid motif RXXR, a pair of arginine residues separated by 2 amino acid residues, is a potential cleavage site for proprotein convertase. The experiments above had indicated that the arginine-rich sequence RRLYKRSRPRSLR promotes tail cleavage, although the short motif RRLYKR alone is sufficient. Because RRLYKRSRPSRLR contains two potential sites for furin-like activity (RRLYKR and RSPR), we next investigated the contribution of individual basic residues in cleavage at these sites. Using site-directed mutagenesis technique, we generated four mutants from the construct C13tail: CR8P, CR9P, CK12A, and CR13S (Fig. 3A). In these constructs, the basic amino acids arginine and lysine are individually mutated to non-basic residues. Expression of these constructs in COS-7 cells and analysis of the product on
Western blot probed by 4B6 indicated that all constructs were equally well expressed (Fig. 3B). Anti-His antibody staining indicated that the uncleaved product of all these mutated constructs was present in the medium at 30–60% of the levels of the C2tail construct (Fig. 3C), which totally lacked the cleavable sequence. This suggested that each individual basic residue in the minimum necessary sequence (RRLYKR) is important for promoting substrate cleavage.

To investigate the effect of the consensus sequence RSPR on product degradation, an RSPR sequence was inserted into the construct C7tail (which was not subject to cleavage in the preceding experiments). This created the construct del8–12, in which the arginines were individually replaced to create delR13S and delR16P (Fig. 3C). PAGE analysis of the EcoRV–ApaI fragments of these three constructs showed that the constructs had the expected sizes (data not shown). Cell lysate and culture medium from COS-7 cells transiently transfected with these three constructs were analyzed on Western blot. Staining with 4B6 detected no difference in expression levels (Fig. 3B). However, when probed with anti-His antibody, the band intensities of del8–12, delR13S, and delR16P in culture medium decreased somewhat indicating that there was some limited cleavage at these RSPR sites (Fig. 3C).

We next investigated the effects of mutating both consensus sequences simultaneously. Mutating one basic amino acid in each consensus sequence generated the construct G3KRTS, which also converted LR to SR as in other constructs. Mutating all the important basic amino acids that may be involved in the tail cleavage resulted in the construct G3allmut (Fig. 4A). The EcoRV–ApaI fragments of these two constructs were slightly larger than that from C2tail, as expected (data not shown). Transfected COS-7 cells expressed these constructs well, as indicated by Western blot development shown below the figure.
sequence RRLYKRRSPRSRLH, which contributes basic residues to both motifs (RRLYKR and RSPR) is not absolutely required for activity, because the KR to TS mutation did not completely eliminate cleavage activity. Mutations of all basic amino acid residue completely inhibited tail cleavage.

**Cleavage of the Consensus Sequence Obtained from Other Aggrecan Species**—Thus far, our studies had focused on chicken aggrecan. However, the aggrecan G3 tail sequence is conserved across rat, human, dog, mouse, cow, and chicken species (Fig. 5A). All of them contain the consensus sequences, (R/H)(R/H)XXBKR and RXXR, for activity, because the KR to TS mutation did not completely eliminate tail cleavage. (Fig. 5B). Only in mouse is histidine substituted for the initial arginine, and only in the bovine sequence is the second arginine replaced by histidine. To investigate if the RRLYKR (in rat and cow) and RHLQKR (in cow) in the former consensus sequence, and RRSSR (rat, human, and dog) and RHPR (in human) in the latter consensus sequence, were involved in tail degradation, these sequences were inserted into the construct C7tail producing HuC8–13, BoC8–13, HuC13–16, and HuC16–19 (Fig. 5C). The EcoRV-Apal fragments from these four constructs were examined in PAGE analysis (data not shown). Products from COS-7 cells transiently transfected with these constructs were analyzed on Western blot probed with 4B6 and anti-His antibody (Fig. 5D). All constructs were equally well expressed as detected with 4B6, and the amount of secreted (uncleaved) product from these four constructs was greatly reduced compared with the uncleavable construct C2tail. This suggests that all individual sequences (RRLQKR, RHLQKR, RRSSR, and RHPR) were quite efficiently cleaved by furin-like activity in these constructs.

**Cleavage of G3-G3 Duplex and Effects of GAG Chains, Protease Inhibitors, and Tail Mutations on the Cleavage**—To study the degradation process at the domain level, two constructs, G3G3 and G3KS, were generated, and transiently expressed in COS-7 cells along with G3His (Fig. 6). G3G3 was composed of two G3 domains joined C-terminal to N-terminal. G3KS consisted of a G3 domain and appended KS sequence. Cell lysate and culture medium were analyzed on Western blot probed with 4B6. G3G3 products in lysate fraction exhibited the size of 72 kDa. However, the majority of secreted product was 48 kDa, a size similar to G3His, suggesting degradation of G3G3 (Fig. 6). It should be pointed out that the size of G3G3 in cell lysate did not double the size of secreted G3. This may be due to incomplete glycosylation of the second G3. Another possibility is that secretion of G3G3 product is a fast step following G3G3 cleavage patterns differed. The product of G3G3 received exo- and endo-RVKR-chloromethylketone was added to the culture medium. Cells transfected with G3G3 construct were incubated with this medium to test the effect of the inhibitor on the cleavage of G3G3 product. At both concentrations (25 and 50 \( \mu \)M), the observed smear suggested GAG chain attachment to the KS sequence. Interestingly, we did not observe secreted product with a size of 48 kDa (G3 fragment), implying that the secreted product of G3KS was not cleaved and suggesting that the presence of the KS domain inhibited proteolysis of an otherwise cleavable tail sequence.

To investigate how the tail was cleaved, furin inhibitor deamoyl-RVKR-chloromethylketone was added to the culture medium. Cells transfected with G3G3 construct were incubated with this medium to test the effect of the inhibitor on the cleavage of G3G3 product. At both concentrations (25 and 50 \( \mu \)M), the inhibitor reduced cleavage of G3G3 product (Fig. 7). Based on the cleavage of G3G3 product, we generated 10 constructs (C2G3, C7G3, C13G3, CR9PG3, CK12AG3, CR13SG3, G3KRTSg3, G3allmutG3, del8–12G3, and HuC8–13G3) containing a mutated or truncated G3 followed by a KS sequence. Interestingly, we did not observe secreted product with a size of 48 kDa (G3 fragment), implying that the secreted product of G3KS was not cleaved and suggesting that the presence of the KS domain inhibited proteolysis of an otherwise cleavable tail sequence.
Cleavage of G3 tail inhibited by glycosaminoglycan chains. Cell lysate and culture medium from G3G3 and G3KS were analyzed on Western blot with G3KRTSG3, each of which contains RRLYKR and RHPR, respectively, appeared to have undergone extensive cleavage (at round 50%). Interestingly, CR9PG3, CK12AG3, CR13SG3, G3KRTSG3, and G3allmutG3, which contains point mutations in the RRLYKR sequence or both RRLYKR and RSPR, still received decent amount of cleavage. These results suggested that some clusters of basic amino acids are involved in the degradation. It should be pointed out that C2G3 and C7G3, which contain no basic amino acids in the tail region, still received some degradation, indicating some non-specific activity. In particular, degradation of C7G3 is unexpectedly severe, a level even higher than some constructs containing basic amino acids in this furin cleavage motif.

RXXR-mediated Cleavage Patterns Are Specific to Aggrecan and Not Observed in Versican—Versican, another member of the large aggregating chondroitin sulfate proteoglycan family, has also been extensively studied. Versican G3 and G3 domains are highly conserved (2, 29, 30). The versican tail also contains an RXXR consensus sequence. To investigate if the tail of versican is also involved in degradation, human (hvG3) and chicken (cvG3) versican G3 domains were joined to aggrecan G3 clones (aG3) to generate hvG3aG3 and cvG3aG3 constructs (Fig. 9A). These constructs were transiently expressed in COS-7 cells, and the products from the cell lysate and culture medium were analyzed on Western blot probed with 4B6. All constructs were equally well expressed and were secreted to culture medium as shown by 4B6 probing (Fig. 9B). However, little degradation of the products was detected in the culture medium, while the control construct aggrecan G3G3 received severe cleavage. The tail sequences of human versican G3 domain and chicken versican G3 domain were modified to contain RSPR sequence (constructs hvRSPRaG3 and cvRSPRaG3), which is identical to that detected in the chicken aggrecan G3 domain. Surprisingly, no tail degradation was detected even with this modification (Fig. 9B), whereas the aggrecan G3 construct containing this sequence (del8–12G3) received notable cleavage (Fig. 8B).

We then examined whether the tail cleavage could be obtained by using chondrocytes. Bovine chondrocytes isolated from caudal discs were grown on 6-well tissue culture plates as monolayer cultures, followed by transfection with the recombinant constructs G3G3, G3allmutG3, cvG3aG3, and hvG3aG3 and the control vector pcDNA3. Product analysis on Western blot probed with 4B6 indicated that similar results were obtained (Fig. 9C). The little discrimination was that the G3G3 product in the culture medium was completely cleaved and that a small amount of the cell-associated G3G3 product was

![Fig. 6. Cleavage of G3 tail inhibited by glycosaminoglycan chains. Cell lysate and culture medium from G3G3 and G3KS were analyzed on Western blot probed with 4B6. The G3G3 products in the lysate had the expected size. However, most of the secreted G3G3 product had a size similar to that of G3His. The faint band at 70 kDa probably represents intact G3G3. The G3KS product also exhibited the expected size, and the smear (the larger size) suggested GAG chain attachment to the KS sequence. No cleavage was observed for G3KS product.](http://www.jbc.org/)

![Fig. 7. The effect of furin inhibitor on tail cleavage. COS-7 cells were transiently transfected with G3G3 construct, and the cultures were maintained in the presence or absence (control) of furin inhibitor decanoyl-RVKR-chloromethylketone (25 and 50 μM), or protease inhibitors Amastatin (50 μM) and E64 (50 μM) for 3 days. Cell lysate and culture media were analyzed on Western blot probed with 4B6. The furin inhibitor reduced cleavage of the G3G3 product.](http://www.jbc.org/)

![Fig. 8. Cleavage of aggrecan G3 tail in constructs lacking the consensus sequence RXXR. A, ten constructs were generated by linking the native G3 cDNA with each of the mutated or truncated G3 constructs, resulting in C2G3, C7G3, C13G3, CR9PG3, CK12AG3, CR13SG3, G3KRTSG3, G3allmutG3, del8–12G3, and huC16–19G3. B, products of these 10 constructs and G3G3 were analyzed on Western blot probed with 4B6. All constructs were well expressed, and the amounts of products in cell lysate were similar in sizes and expression levels as expected (top). Detection of two bands in culture medium suggested that all products were cleaved after secretion (bottom). Notably, however, the G3G3 control underwent 95.8% cleavage while mutants exhibited lower cleavage levels. The densitometry of both bands and percentage of cleavage were given below the gel.](http://www.jbc.org/)
cleaved as compared with those expressed by COS-7 cells.

To ensure that the native tail of versican G3 was not cleaved, we expressed aggrecan G3 construct (G3His) and versican G3 constructs. We also engineered a His-tag at the end of the G3 domain and added a leading peptide of link protein at the N-terminal. This leading peptide serves as a signal sequence for product secretion and contains an epitope recognized by the monoclonal antibody 4B6. With this method, we were able to detect the His-tag epitope in the secreted product of a G3 construct was not detected in culture medium using antibody against a C-terminal His-tag epitope but could be detected in cell lysate. An antibody against an epitope (4B6) at the N-terminal region detected product in both culture medium and cell lysate. Hence, we hypothesized that the G3 tail cleavage is associated with product secretion. The present study was designed to test this hypothesis.

Because the tail consists of only 20 amino acids, the cleaved tail is too small to be detected. As well, removal of the tail from the G3 domain reduces the size of the domain only slightly. This makes it difficult to observe the phenomenon. Glycosylation of the G3 domain obscures the effect even more. To overcome these problems, we used incremental deletion and site-directed mutagenesis techniques to generate a large number of constructs. We also engineered a His-tag at the end of the G3 domain and a leading peptide of link protein at the N-terminal. This leading peptide serves as a signal sequence for product secretion and contains an epitope recognized by the monoclonal antibody 4B6. With this method, we were able to demonstrate that two tandem boxes, RRXXK and RXXR, were the consensus sequences and involved in tail cleavage. Inclusion of the last 9 amino acids of chicken aggrecan in the constructs did not affect the results, indicating these 9 amino acids are not involved. Further confirmation was obtained by replacing these 9 amino acids with 9 unrelated amino acids. It should be pointed out that the results were reproducible, but they were not clear-cut, because the extent of cleavage was variable. This might be affected by culture and transfection conditions. It might also be affected by the sensitivity of the Western blot detection method, although we standardized our methods. Because we isolated and used at least two clones for transfection, these varying factors do not affect the interpretation of our results.

The structure of the aggrecan tail is highly conserved. Among rat, human, dog, mouse, and cow, there is 80% homology. The homology of the consensus sequences, (R/H)(R/H), is even greater. Although the sequence of chicken aggrecan tail is relatively different from those discussed above, the chicken aggrecan tail does contain the consensus sequences, RRXXK and RXXR. Using this knowledge, we were able to pinpoint these sequences as essential for cleavage. We confirmed this by testing the effects of these motifs in aggrecan from other species. Because the tail structure of aggrecan is highly conserved, our results do not exclude the possibility that other amino acids may also play a role in tail cleavage. As discussed below, the tail cleavage is very complex.
Proprotein convertases are a group of proteases that cleave sequences composed of pairs of basic amino acids. It has been reported that the minimum required site for convertase cleavage is RXRX (36–38). Proprotein convertases often act in the trans-Golgi and on cell surfaces. Cleavage is often the step that converts a protein or peptide to its active form. Because the aggrecan C-terminal tail contains many basic amino acids and RXRX sites, we thus designed experiments to examine whether these sites are cleaved during product processing. We used the chicken aggrecan as it contains only one RXRX.

Although the site-directed mutagenesis technique allowed us to confirm that the consensus sequences were important in tail cleavage, it led to a puzzling situation. For example, in the seven constructs listed in Fig. 3A (CR8P, CR9P, CK12A, CR13S, del8–12, delR13S, and delR16P), we initially expected to detect tail cleavage in del8–12 construct, because it does contain RXRX. However, we did not observe a convincing difference between del8–12 and the rest. Given that a construct completely lacking a consensus sequence was degraded somewhat, it seems likely that other amino acids in the tail, especially other basic amino acids, play a role in nonspecific degradation. Despite this, our studies do indicate that the consensus sequences were important: This interpretation was supported by the results obtained from two other constructs (G3KRTS and G3allmut). G3KRTS contains mutations in both consensus sequences in the tail. Product analysis indicated that G3KRTS was still severely degraded. On the other hand, when all basic amino acids in both consensus sequences were altered, as in the construct G3allmut, the resulting product received no degradation.

To clarify this issue, we sought to develop a better method for studying tail cleavage. A construct containing two G3 domains was then produced. This G3G3 product was cleaved in the middle producing a smaller fragment equal to G3 that could be detected by 4B6. This G3G3 construct thus served as an ideal model to identify the exact site of tail cleavage. It should be pointed out that in the G3G3 construct, the tail cleavage is not complete in COS-7 cells: ~96% of the products were cleaved. The intact product was detected in culture medium using the 4B6 antibody but not with the anti-His antibody. The former antibody is more sensitive. Intact G3 with His-tag was also successfully purified from culture medium with nickel-nitrilotriacetic acid beads (11, 13). It may be that a small proportion of G3 is kept intact in specific interactions. However, in the case of chondrocyte transfection, the G3G3 product in culture medium was completely cleaved. Perhaps, the proteolytic activity in chondrocyte culture was more active. Furthermore, the cell-associated fraction contained a small portion of cleaved G3 product. It is possible that the cleaved G3 fragment interacted with cell surface proteins. The CRD and CBP motifs of aggrecan G3 domain are structurally similar to the CRD and CBP motifs of versican G3 domain, which have been shown by us to bind to cell surface and β1 integrin is involved in this interaction (39, 40). Our experiments indicate that, although G3 tail cleavage occurred in both COS-7 cells and chondrocytes, there were some differences between these two types of cells in the extent of cleavage. Because our study was performed in a non-chondrocyte model system, to what extent this tail cleavage occurs during the normal processing of aggrecan in cartilage is unclear at present.

Using the G3G3 duplex as a model, we were able to demonstrate that tail cleavage does not occur prior to product secretion, because the G3G3 product from cell lysate migrated almost exclusively as a single band. We occasionally observed a very faint band in the G3G3-transfected cell lysate that migrated at a position equal to G3. This could be a result of the interaction of secreted G3 with the cell surface during lysate or post secretory G3 bound to the cell surface, although the cells were rinsed with PBS before harvest. The possibility that the CRD motif binds to the GAG chains on the cell surface is not excluded.

In addition to confirming our initial observations, the G3G3 model also revealed the motifs necessary for tail cleavage. For example, HuC16–19G3 (containing RSPR) received more cleavage than del8–12G3 (containing RSPR) did. Using the G3G3 strategy, we observed that the products of C2G3, C7G3, and G3allmutG3 received trace amounts of degradation, which could not be previously detected by using the constructs C2, C7, and G3allmut. The C2G3 contains no tail sequence but still received trace amounts of degradation producing a faint band with of G3 size after overexposure. It appears that the one or more enzymes responsible for the tail cleavage not only recognize the tail sequence but may also recognize the C terminus of the CBP motif. Because no known proprotein convertase has such a function, it is likely that there may be a new protease involved in aggrecan tail cleavage. Although we have demonstrated that furin-like protease inhibitors were able to reduce the cleavage, the nature of the protease(s) involved in this action is still not known.

The idea that basic amino acids are important in tail cleavage was further supported by experiments involving G3KS product. G3KS contains the G3 domain joined to a fragment of keratan sulfate modifying sequence (KS domain). The product of G3KS was not cleaved. Although there was no direct evidence that keratan sulfate chains were added to the KS-modifying sequence in these cells under these conditions, the increase in size of product in culture medium and diffusion patterns of bands suggested modification by GAG chains. Because the GAG chains contain a high density of negative charge, our results may imply that the GAG chains protect the cleavage site either sterically or electrostatically (or by a combination of effects).

Our study has shown that the cleavage mechanism examined here is specific to aggrecan. Versican, another member of the large aggregating chondroitin sulfate proteoglycan family, also contains a G1 domain, a G3 domain, and a CS sequence for GAG modification. The structures of the G1 and G3 domains are very conserved between aggrecan and versican. Analysis of the products from chicken and human versican G3 domains indicated that the tail in versican was not cleaved. Because both versican and aggrecan G3 domains are processed in a similar fashion, the difference in tail cleavage suggests additional roles for the tail. Sequence alignment indicated that the tails are different between aggrecan and versican. There is a major difference in the sequence upstream of the “furin-cleavage area” in the C-terminal tail of aggrecan and versican. Versican has an “insert” (KYFKNSSSADDNSINTSKHDH) between the PSSYQR motif and the furin-cleavage motif RXRX, which is absent from aggrecan. This insert, although eliminating the RRLYKR motif present in aggrecan, instead puts TSKHDH upstream of the RXRX motif, which may be a “pseudo” furin site. Although RXRX is the minimal requirement for furin recognition, additional Arg or Lys around this recognition motif is important for furin cleavage (41, 42).

In conclusion, the studies reported here have revealed for the first time that the carboxyl tail of the aggrecan G3 domain is subject to cleavage. Two consensus sequences RRXXK and RXRX are involved in the cleavage, which appears to be specific to aggrecan. These findings may have implications for the future development of therapies for the loss of aggrecan function associated with aging and arthritis.
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