Aggrecan is the major proteoglycan in the extracellular matrix of cartilage. A notable exception is nanomelic cartilage, which lacks aggrecan in its matrix. The example of nanomelia and other evidence leads us to believe that the G3 domain plays an important role in aggrecan processing, and it has indeed been confirmed that G3 allows glycosaminoglycan (GAG) chain attachment and product secretion. However, it is not clear how G3, which contains at least a carbohydrate recognition domain (CRD) and a complement binding protein (CBP) motif, plays these two functional roles. The present study was designed to dissect the mechanisms of this phenomenon and specially 1) to determine the effects of various cysteine residues in GAG modification and product secretion as well as 2) to investigate which of the two processing events is the critical step in the product processing. Our studies demonstrated that removal of the two amino-terminal cysteines in the CRD motif and the single cysteine in the amino terminus of CBP inhibited secretion of CRD and CBP. Use of the double mutant CRD construct also allowed us to observe a deviation from the usual strict coupling of GAG modification and product secretion steps. The presence of a small chondroitin sulfate fragment overcame the secretion-inhibitory effects once the small chondroitin sulfate fragment was modified by GAG.

Proteoglycans are a family of glycoconjugates with a central core protein to which the glycosaminoglycan (GAG) side chain(s) is covalently linked post-translationally (1). The functions of proteoglycans are due in large part to their GAG chains (2, 3). These GAG chains are acidic and participate in a wide variety of interactions with other matrix macromolecules, cations, and water (4, 5). They can sequester a variety of extracellular proteins at cell surfaces. In cartilage, the molecules that make up the extracellular matrix include proteoglycans, hyaluronan, type II collagen, and glycoproteins. Aggrecan is the major structural proteoglycan in cartilage and is responsible for its resilience and load-bearing properties. Loss of aggrecan is a major feature of cartilage degradation associated with arthritis (6, 7).

The core protein of aggrecan is composed of three globular domains (G1, G2, and G3) with a large extended region between G2 and G3 for GAG chain attachment, CS (1, 8, 9). G1 comprises the amino terminus of the core protein. This domain has the same structural motif as link protein (10). G2 is homologous to the tandem repeats of G1 and of link protein. G3, which makes up the carboxyl terminus of the core protein, is composed of folded modules including alternatively spliced epidermal growth factor-like motifs, a carbohydrate recognition domain (CRD), a complement binding protein (CBP)-like domain, and a short carboxyl-terminal tail (11).

The G3 domain seems to be important in aggrecan processing. This was initially observed in chicken nanomelia, in which a point mutation produces a premature stop codon on the amino-terminal side of G3 (12). This truncated core protein generates a lethal phenotype in homozygous form (failure of chondrogenesis and osteogenesis) and dwarfism in heterozygous form (12). In the nanomelic mutation, the cartilage matrix lacks aggrecan. The mutation seems to inhibit aggrecan secretion to the matrix, and no modification by GAG occurs (13, 14). It has been proposed that G3 may regulate the attachment of GAG chains and affect the secretion of aggrecan, and it was later demonstrated that G3 is important in product secretion (15). Recently, data from independent studies indicated that the G3 domain of aggrecan and also versican plays an important role in GAG chain attachment and product secretion (16–21). The question remains, is it possible to distinguish which step is more important in the process?

The present study was designed to dissect the mechanisms governing the effects of the G3 domain in GAG chain attachment and product secretion. We generated a large number of deletion mutants of the G3 domain to investigate whether G3 conformation, through the formation of cysteine-mediated disulfide bonds, was involved. We determined that cysteines present in G3 do indeed play a role in product secretion. We also used mutants to distinguish G3 structural elements that allow product secretion without enhancing GAG chain attachment and vice versa.
**TABLE I**

| Primer         | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| CRBmuC1XhoI   | 5'–GGG CCC TCA GAG TGG GAG GAA GGC TGG ATC AAG TCC CAG GGC CAC GGC       |
| CRBmuC2XhoI   | 5'–GGG CCC TCA GAG TGG GAG GAA GGC TGG ATC AAG TCC CAG GGC CAC GGC       |
| CRDmuC1+2XhoI | 5'–GGG CCC TCA GAG ATC AAG TCC CAG GGC CAC GGC                           |
| CRDmuC1XhoI   | 5'–GGG CCC TCA GAG ATC AAG TCC CAG GGC CAC GGC                           |
| CRDmuC2XhoI   | 5'–GGG CCC TCA GAG ATC AAG TCC CAG GGC CAC GGC                           |
| CRDmuC4XbaI   | 5'–GGG TCT AGA ATG CCA GAT CAT CAC AAC GAC GTC                           |
| CRDmuC5XbaI   | 5'–GGG TCT AGA CTT GCA CTT ATG ATG AAG GAG GGG GGG GGG GGG GGG GGG       |
| CRDmuC6XbaI   | 5'–GGG TCT AGA CTT GCA CTT ATG ATG AAG GAG GGG GGG GGG GGG GGG GGG       |
| CRDmuC6XbaI+3 | 5'–GGG TCT AGA CTT GCA CTT ATG ATG AAG GAG GGG GGG GGG GGG GGG GGG       |
| CRDXhoI       | 5'–AAA AAA TTC AGA GGT ATG ATG ATG ATG AAC TGT TTC CTT GTC GCA           |
| CRDCXbaI      | 5'–AAA AAA TTC AGA GGT ATG ATG ATG ATG AAC TGT TTC CTT GTC GCA           |
| CRBPmuC1XbaI  | 5'–GGG GCC TCA GAC GGC TGG GGG GAC CCA CCT GAA                           |
| CRBPmuC3XbaI  | 5'–GGG TCT AGA GCA GGA TAT CCG CCG TTC CTC CCA GGC CCT GTT TGG CCA       |
| CRBPmuC4XbaI  | 5'–GGG TCT AGA GCA GGA TAT CCG CCG TTC CTC CCA GGC CCT GTT TGG CCA       |
| CRBPmuC5+4XbaI| 5'–GGG TCT AGA GCA GGA TAT CCG CCG TTC CTC CCA GGC CCT GTT TGG CCA       |
| CBPCXbaI      | 5'–AAA AAA TTC AGA GTT GGT GGA GAC CCA GCA                               |
| G3XbaI        | 5'–AAA TTC AGA GGT ATG ATG ATG ATG AAC TGT TTC CTT GTC GCA GCA           |

**EXPERIMENTAL PROCEDURES**

**Materials—** Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were purchased from Roche Molecular Biochemicals or Invitrogen. Bacterial growth medium was from Difco. Prestained protein markers were from New England Biolabs. Lipofectin, Genetecin (G418), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, Hanks’ balanced salt solution, and trypsin/EDTA were from Invitrogen. The ECL Western blot detection kit was from Amersham Biosciences, Inc. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Sigma. The DNA mini-prep kits and midi-prep kits were from Qiagen Inc. Tissue culture plates (12-well, 6-well, and 100 mm) were from Nunc Inc. All chemicals were from Sigma. COS-7 cells, from American Type Culture Collection, were cultured in DMEM supplemented with 5% fetal bovine serum at 37 °C in a humidified incubator containing 5% CO2.

**Strategy for Generation of Recombinant Constructs**—In this study, a total of 32 recombinant constructs were used. Of these, 22 (including the G3 construct) were generated based on an aggrecan G3 construct, which was cloned and expressed in our previous studies (22, 23). This construct contains the leading peptide, which is composed of a link peptide signal sequence and an epitope recognized by the monoclonal antibody 4B6 (24), and the aggrecan G3 domain with the carboxyl tail, which was cloned and expressed in our previous studies (22, 23). This construct contains the leading peptide, which is composed of a link peptide signal sequence and an epitope recognized by the monoclonal antibody 4B6 (24), and the aggrecan G3 domain with the carboxyl tail, which was cloned and expressed in our previous studies (22, 23). The leading peptide and G3 domain are linked by a restriction endonuclease site, whereas the tail and the vector are linked by an XhoI site. Individual mutants were generated using PCR. All names of the primers are given in Fig. 1, and the oligonucleotide sequences are listed in Table I. The location of primers and the presence or absence of cysteines are also given in Fig. 1. All 5’ primers contained the XhoI restriction site, and 3’ primers contained the XbaI restriction site, allowing insertion of the PCR-derived fragments into XhoI- and XbaI-digested G3 construct. Thus, the G3 domain of the G3 construct was replaced with the PCR-generated fragments. Twenty such constructs are given in Fig. 1, whereas the constructs G3 and CRDCBP (generated by primers CRDXXhoI and CBPCXbaI) are given in Fig. 5.

The remaining 10 constructs were based on a G1CSD3 construct originally described by us (21). Also known as “mini-aggrecan,” this construct contains the leading peptide (for antibody recognition), the aggrecan G1 domain, a small CS fragment (CSD) containing amino acids 1274–1362 of chicken aggrecan and 8 consensus sequences for GAG chain modification (9), the aggrecan G3 domain, and a short carboxyl-terminal tail. To create the mutants, various domains were deleted (Fig. 5) or swapped with CRD and CBP domains containing mutated cysteines (Fig. 6). Because the G3 domain in G1CSG3 contains a 5’ XhoI site and a 3’ XbaI site, the CRDCBP fragment was synthesized with the primers CRDXXhoI (containing the XhoI site) and CBPCXbaI (containing the XbaI site). All other fragments were obtained from some of the 20 constructs described above (also containing XhoI and XbaI). DNA Manipulation and Clone Selection—DNA was amplified in PCR 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 (Roche Molecular Biochemicals). The reactions were carried out at 94 °C for 3 min for 1 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 and then doubly digested with two appropriate restriction endonucleases. The DNA was ligated into the appropriate linearized plasmids (e.g., pcDNA3). A ligation mixture typically contained 2 µl of 5× ligation buffer, 1 µl of T4 DNA ligase, 3 µl of plasmid vector (50 ng), and 4 µl of insert fragment (150 ng). The ligation reaction was carried out at 16 °C overnight. Five µl of the ligation mixture was used for heat shock transformation of competent Escherichia coli strain DH5α. Bacteria were then transferred to 500 µl of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM MgCl2, 20 mM glucose, pH 7.0), agitated at 225 rpm for 1 h at 37 °C, spread onto LB agar plates containing 100 µg ampicillin/ml, and cultured at 37 °C overnight. The identities of 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 sequences (9, 12).

**Construct Expression and Protein Analysis—**To analyze gene expression, COS-7 cells were transiently transfected with recombinant constructs using Lipofectin (25) according to the manufacturer’s instructions (Invitrogen). Briefly, the cultured COS-7 cells were seeded on 12-well tissue culture plates (1.5 × 105 cells/well). The cells were allowed to attach and grow overnight in DMEM containing 5% fetal bovine serum. Cells were transfected once they reached 70% confluence. Lipofectin (0.5 µl) was incubated with plasmid DNA (~2 µg) for 15 min in 100 µl of DMEM followed by an addition of 900 µl of DMEM. Concurrently, COS-7 cell 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 an incubator. The DNA–Lipofectin mixture was then incubated with 1 ml of DMEM containing 5% fetal bovine serum. The cells were harvested in 400 µl of lysis buffer 3 days later, and samples of cell lysate and culture medium were frozen at −70 °C until analysis.

Cell lysate and culture medium samples were prepared in loading dye and subjected to SDS-PAGE in separating gel containing 5 or 7% acrylamide. Two layer gels (12% and 5%) were used if large and small products were analyzed in the same experiment. The buffer system was Tris-glycine buffer according to Amresco containing 1% SDS. Separated proteins were trans-blotted onto a nitrocellulose membrane in 1× Tris-glycine buffer containing 20% methanol at 60 °V for 2 h at 4 °C. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk powder (TBSTM) for 30 min at room temperature and then incubated at 4 °C overnight with the monoclonal antibody 4B6 (or biotinylated 4B6) in TBSTM. The membranes were washed with TBST (3 × 10 min washes) and then incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:10,000 dilution in TBSTB). After washing as above, the bound antibodies were visualized with chemiluminescence (ECL kit, Amersham Biosciences, Inc.).

**Protein Precipitation—**Trichloroacetic acid was added to culture medium or cell lysate to a final concentration of 10% followed by incubation on ice for 30 min. The precipitated proteins were pelleted by centrifugation (10,000 × g) at 4 °C for 30 min. The pelleted proteins were resuspended in loading dye and subjected to Western blot analysis as above.

**Chondroitinase ABC Treatment—**Protein G beads (50 µl of gel slurry) were incubated with excess 4B6 antibody at room temperature for 2 h. The unbound antibody was removed, and the gel beads were...
RESULTS

Cysteines in the G3 Domain Are Involved in Product Secretion—To study the effects of cysteines in the aggrecan G3 domain on product secretion, we tested 20 constructs in which the cysteines were subjected to mutagenesis in different combinations. There are six cysteines in the CRD motif and four in the CBP motif. Three groups of mutation constructs in CRD were generated, resulting in 12 recombinant constructs (Fig. 1). In Group I constructs, the three cysteines in the amino-terminal fragment (C1 to C3) and the three cysteines in the carboxyl-terminal fragment (C4 to C6) of CRD motif were mutated in parallel. The four resulting constructs, CRD-C2C3C4C5, CRD-C3C4, and CRD-C0, retained, respectively, all cysteines, the middle four cysteines, the middle two cysteines, and no cysteines. In Group II, the three cysteines in the carboxyl-terminal fragment of CRD were deleted, and the three cysteines in the amino-terminal fragment were mutated, resulting in four constructs, CRDC1C2C3, CRDC1C3, CRDC2C3, and CRDC3. In Group III, the three cysteines in the amino-terminal fragment of CRD were deleted, and the three cysteines in the carboxyl-terminal fragment were mutated, producing constructs CRDC4C5C6, CRDC4C6, CRDC4C5, and CRDC4.

The amino acid sequences of all constructs listed in Fig. 1 are given in Fig. 2. All amino acid sequences were deduced from the cDNA sequences after the constructs were generated. We isolated at least two clones from each expression construct to guarantee that we would obtain a sequence that was identical to the reported aggrecan sequences (11, 12). The underlined amino acids (Gly or Trp) were mutated from the wild-type Cys residue. These two amino acid residues were chosen to replace Cys because of codon similarities.

The 12 constructs obtained from the CRD motif were expressed in COS-7 cells. Culture medium and cell lysates were harvested and analyzed by electrophoresis and Western blot as described under “Experimental Procedures.” Probing with the monoclonal antibody 4B6 showed that all expressed constructs were of the expected sizes (Fig. 3). In Group I, all constructs were well expressed and secreted except CRDC3C4, which was poorly secreted. The CRDC3C4 product was well detected in 10-fold concentrated culture medium (Fig. 3 A). In Group II, CRDC1C2C3, CRDC1C3, CRDC2C3, and CRDC3 were well expressed, but only CRDC1C2C3 and CRDC1C3 were well secreted. The products of CRDC2C3 and CRDC3 were poorly secreted as compared with the control construct CRD. The secreted products of CRDC2C3 and CRDC3 were visible on Western blot after these samples were concentrated (10-fold) (Fig. 3 B). All Group III products were well expressed and secreted to culture medium as compared with the control CRD (Fig. 3 C).

The G3 complement binding protein-like motif contains four cysteines. To study their effects on product secretion, we generated two more groups of constructs (Group IV and Group V, Fig. 1). In Group IV, two cysteines in the carboxyl-terminal fragment of CBP were mutated, producing CBPC1C2C4 and CBPC1C2. These constructs were well expressed and well secreted (Fig. 4 A). Expression of the CBP construct or CBP containing the carboxyl-terminal tail (CBP-tail) indicated that the tail is not required for product secretion (Fig. 4 A).

In Group V, the amino-terminal cysteine of CBP was mutated along with the two cysteines in carboxyl fragment, producing constructs CBPC2C3C4, CBPC2C4, CBPC2C3, and CBPC2. Culture medium and cell lysates from COS-7 cells transfected with these constructs were analyzed on a Western blot probed with 4B6. The product of CBP-tail was used as a control. All constructs were weakly detected in cell lysates and culture medium as compared with CBP-tail (Fig. 4 B). After product precipitation (10-fold concentration), they were readily detected on Western blot (Fig. 4 C).

The Effects of G3 on GAG Chain Attachment and Product Secretion Represent Two Separate Functions; GAG Chain Attachment Enhances Secretion but Not Vice Versa—In addition to investigating the effects of G3-subdomain conformation on construct secretion, we aimed to dissect the functions of G3 in GAG chain attachment and product secretion. We did not observe accumulation of GAG chain-modified product in cell lysate; GAG-modified products were always secreted to culture medium, indicating the close relationship of these two processes. We have previously shown that the construct G1CSD is not modified by GAG chain and not secreted, whereas G1CSDG3 is modified by GAG chains, and the product is secreted (21). We thus sought to identify element(s) in G3 that facilitate product secretion but had no effect on GAG chain attachment and elements that are not secreted on their own but enable GAG chain attachment. The current study indicated that the products of G3 subdomain constructs CRD and CBP-tail were well secreted. We thus examined these fragments for GAG chain attachment by adding them to the G1CSD construct. All new G1CSD-linked constructs, now named G1CSDCRDCBP, G1CSDCRD, and G1CSDCBP-tail, as well as G1CSDG3 and G1CSD controls (Fig. 5 A), were well expressed (Fig. 5 B). Interestingly, the G1CSDCBP-tail product was not modified by GAG chains nor secreted to culture medium, although CBP-tail, in the absence of G1CSD domains, is secreted on its own. On the other hand, the products of G3 and CRDCBP (Fig. 5 C) and the product of CRD (Fig. 3 A) were well expressed and secreted, and constructs containing these fragments (i.e. G1CSDG3, G1CSDCRDCBP, and G1CSDCRD) generated products modified by GAG chains and secreted. It was noted that the cell-associated products of the above five constructs containing the CSD fragment showed little diffusion, likely indicating low levels of GAG chain modification. The apparent molecular weights of products in cell lysate (lys) and culture medium (med) were much greater than the calculated molecular mass (cal) of the recombinant constructs (Fig. 5 D). Although the apparent molecular mass of the cell lysate products was greater than the calculated molecular mass, a lack of diffusion implied that these products were not subjected to GAG addition. Rather, they may have been modified by glycosylation, as these constructs contain several potential sites for N-glycosylation (located at Asn residues 76, 122, 330, and 2082) according to the published sequence (12). Thus, all constructs containing the CBP motif have four potential sites for glycosylation, whereas constructs containing the G1 domain have three potential sites for glycosylation. Because glycosylation of these recombinant constructs appeared not to be affected by the presence of different G3 subdomains, no further study was conducted.

In contrast to the cell lysate products, the products in the culture medium migrated diffusely on Western blot, resulting in broad ranges of molecular mass. This may have been caused by GAG chain attachment to core protein, as the core proteins of the recombinant constructs contains eight potential sites for GAG chain modification. To confirm the presence of GAG...
chains, we treated these products with chondroitinase ABC. Treated products migrated as single bands on Western blot, confirming this explanation (Fig. 5E). The addition of GAG chains appeared to be affected by the addition of different G3 subdomains.

The role of the cysteines in the CRD and CBP motifs was further studied by adding the G1CSD fragment to mutant constructs CRDC0, CRDC3, CRDC4, CBPC2, and CBPC1C2 used in the experiments described above. These constructs were labeled G1CSDCRDC0, G1CSDCRDC3, G1CSDCRDC4, G1CSDCRDC5, and G1CSDCRDC6.

**Fig. 1.** Aggrecan CRD and CBP constructs in which cysteines are deleted or mutated. Three groups of constructs containing mutations in CRD and two groups of constructs containing mutations in CBP of aggrecan G3 domain, a total of 20 constructs, were generated. In Group I constructs, the three cysteines in the amino-terminal fragment and the three cysteines in the carboxyl-terminal fragment of the CRD motif were mutated in parallel, resulting in four constructs named CRD, CRDC3C4C5, CRDC3C4, and CRDC0 (where CRD is nonmutant and CRDC0 is a CRD mutant lacking all cysteines). In Group II, the three cysteines in the carboxyl-terminal fragment of CRD were deleted, and the three cysteines in the amino-terminal fragment were mutated, resulting in four constructs named CRDC1C2C3, CRDC1C3, CRDC2C3, and CRDC3. In Group III, the three cysteines in the amino-terminal fragment of CRD were deleted, and the three cysteines in the carboxyl-terminal fragment were mutated, resulting in CRDC4C5C6, CRDC4C6, CRDC4C5, and CRDC4. In Group IV, the three cysteines in the carboxyl-terminal fragment of CBP were deleted, resulting in CBP, CBPC1C2C4, and CBPC1C2. A CBP containing the carboxyl-terminal tail (CBPtail) was included in this group. In Group V, mutations were generated in the cysteine of the amino terminus of CBP as well as the two cysteines in the carboxyl-terminal fragment, resulting in CBPC2C3C4, CBPC2C4, CBPC2C3, and CBPC2. A leading peptide obtained from link protein (nucleotides 1–180) was added to all constructs. The numbers above the schematic correspond to nucleotides in the sequence of full-length aggrecan. The numbers below the schematic correspond to the numbers of amino acids between each two cysteines.

| Group | Construct | Structure |
|-------|-----------|-----------|
| I     | CRD       | CRD       |
|       | CRDC2C3C4C5 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC3C4 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC0 | CRDmuC4 |
| II    | CRDC1C2C3 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC1C3 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC2C3 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC3 | CRDmuC2, CRDmuC3, CRDmuC4 |
| III   | CRDC4C5C6 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC4C6 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC4C5 | CRDmuC2, CRDmuC3, CRDmuC4 |
|       | CRDC4 | CRDmuC2, CRDmuC3, CRDmuC4 |
| IV    | CBPtail  | CBP       |
|       | CBP      | CBP       |
|       | CBPC1C2C4 | CBPmuC3, CBPmuC4 |
|       | CBPC1C2 | CBPmuC4 |
|       | CBPC2C3C4 | CBPmuC3, CBPmuC4 |
|       | CBPC2C4 | CBPmuC3, CBPmuC4 |
|       | CBPC2C3 | CBPmuC3, CBPmuC4 |
|       | CBPC2 | CBPmuC4 |
| V     | CBPC2C3C4 | CBPmuC1, CBPmuC4 |
|       | CBPC2C4 | CBPmuC1, CBPmuC4 |
|       | CBPC2C3 | CBPmuC1, CBPmuC4 |
|       | CBPC2 | CBPmuC1, CBPmuC4 |
Before the addition of G1CSD, the mutant CRDC3 was notably ill-secreted (Fig. 3); with the addition of CRDC3 to G1CSD, the new construct (G1CSDCRDC3) was well expressed, and the products were modified by GAG chains (as indicated by diffusion of the secreted products) and secreted to culture medium (Fig. 6B). The cell-associated products showed little diffusion of bands (little GAG chain modification). Again, the apparent molecular masses of the products in cell lysate (lys) and culture medium (med) were much greater than the calculated molecular mass (cal) of each recombinant construct (Fig. 6C). Chondroitinase ABC treatment further confirmed that the secreted recombinant proteoglycans were subjected to post-translational modification by GAG chains as expected (Fig. 6D).

Taken together, we have studied five types of G1CSD-containing constructs differing in GAG chain modification and product secretion (Figs. 5 and 6). All results are summarized in Table II. (i) Without any components from the G3 domain, the product of G1CSD was not modified by GAG chains nor secreted. (ii) The addition of certain elements from the G3 domain to the G1CSD construct, including G3, CRD, CRDCBP, CRDC0, CRDC4, and CBPC1C2, allowed GAG chain modification and product secretion. All of these G3 elements, when linked to the leading peptide, were well expressed and secreted. (iii) The construct CRDC3 was well expressed but poorly secreted, but the addition of the CRDC3 fragment to G1CSD allowed GAG modification and product secretion. (iv) The construct CBPC2 is poorly expressed and poorly secreted, but the addition of CBPC2 to G1CSD allowed GAG modification and product secretion. (v) The addition of CBPtail to G1CSD facilitated neither GAG modification nor product secretion, although the product of CBPtail was well secreted. The fragments that behaved differently in product secretion when linked with or without G1CSD were further examined in a single experiment. Products from COS-7 cells transiently transfected with CRDC3, G1CSDCRDC3, CBPC2, G1CSDCBPC2, CBPtail, and G1CSDCBPtail were analyzed on Western blot probed with 4B6. The results confirmed that both CRDC3 and CBPC2 fragments enabled GAG addition, and the resulting products, G1CSDCRDC3 and G1CSDCBPC2, were well secreted (Fig. 7). With the attachment of CBPtail, the resulting product G1CSDCBPtail was not modified by GAG nor secreted.

We have thus demonstrated the positive and negative coupling of GAG modification and product secretion; if a fragment facilitates GAG modification to a CS sequence, the resulting product will be secreted, and if a CS fragment is not modified by GAG chains, the product will not be secreted. More importantly, however, the CRDC3 and CBPC2 fragments are the first reported deviation from this coupling. On their own, they are poorly secreted. Only with the addition of the G1CSD fragment and concomitant GAG addition are these fragments secreted. This indicates the primary of GAG modification in the processing of these products.

**DISCUSSION**

This study was designed to investigate the roles of aggrecan G3 domain in GAG chain attachment and product secretion. It is well accepted that the G3 domain is essential for GAG chain attachment and product secretion based on several previous findings. It was initially observed that the chicken mutant nanomelia lacks aggrecan in its cartilage matrix (13, 15, 26),

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**Table II.** Acceptor sites for the recombinant constructs. The upper diagram is the structure of all constructs, which contains the link protein leading peptide (LP) and a fragment of CRD or CBP. The leading peptide and the fragments are linked by the restriction endonuclease site XhoI, and the fragments and the vector are linked by the restriction site XbaI. The amino acid sequences of each fragment and the leading peptide are listed. The cysteine residues and the mutated amino acids (replacement of cysteines) are underlined. The calculated molecular mass of the core proteins (cal), and the apparent molecular mass of the products in cell lysate (lys) and culture medium (med) are shown.
since the nanomeric aggrecan is not modified by GAG chains nor secreted to the matrix due to lack of G3 domain in aggrecan core protein (12). It was later shown that chicken aggrecan G3 domain is secreted at much higher levels than G1, but secretion is not coupled to xylosylation or GAG chain attachment (16, 17, 27). This group also demonstrated that chicken aggrecan G3 domain facilitates G1 secretion, and the central portion of CRD domain is necessary and sufficient for G3 secretion (17). Day et al. (18) generated recombinant constructs containing a CS fragment and different motifs of the G3 domain of human aggrecan and showed that each folded module of the G3 domain allows GAG chain attachment and product secretion. Recently, it has been demonstrated that the central portion of the G3 domain is required for GAG chain attachment and product secretion (19).

In our studies, we demonstrated that GAG chain attachment is an important step in G3 facilitation of G1 secretion, as GAG chain modification enhances product secretion (21). The G3 domain of versican also enhances GAG chain attachment and product secretion, and the CBP motif plays a greater role in enhancing GAG chain attachment than does the CRD motif (20).

At first glance, the above results from different laboratories may seem contradictory. However, these results are easily reconciled as follows. It is reported that G3 secretion is not coupled to xylosylation of contiguous GAG chain consensus sites (16), but our recent studies indicate that GAG chain attachment is required for secretion of a mini-aggrecan construct (containing the G1 domain, a fragment of the CS sequence, and the G3 domain) (21). In the former case, the construct contained only a CS fragment and a G3 domain and lacked a G1 domain (16). We now know that the G1 domain plays an important role in the inhibition of product secretion (20, 21). The G3 product is always well secreted, and this process is not affected by the addition of a CS sequence with GAG chain attachment in normal Chinese hamster ovary cells or a CS sequence without GAG chain attachment in mutant Chinese hamster ovary cells incapable of GAG chain attachment (16). It appears that the secretory property of the G3 domain is not affected by the CS fragment in the CSG3 construct (16). In the latter case, the construct contains a G1 domain, which inhibits product secretion, a CS fragment, and a G3 domain, which enhances product secretion (20, 21). Most significantly, we now know that a construct containing aggrecan G1 and G3 domains is poorly secreted (21). Thus, in a construct such as mini-aggrecan, which contains both a G1 and a G3 domain, a CS fragment with attached GAG chains is needed to overcome G1-mediated inhibition of product secretion (21).

It is reported that G3 facilitates G1 secretion (17). In our previous study, however, the product of a construct containing G1 and G3 domains was weakly detected in culture medium, whereas the product of a construct containing the G1 domain,
a CS fragment, and the G3 domain was readily detected in culture medium (21). Because GAG chain modification also enhances product secretion, and the construct in the former case contains a CS fragment (17), it may be more precise to say that G3 facilitates G1 secretion in the presence of a CS fragment that is modified by GAG chains.

It is reported that exon 15, encoding the central portion of CRD motif, is necessary and sufficient for GAG chain attachment and product secretion (17, 19). It has also been reported that an unfolded GS fragment required one folded protein module (from the G3 domain) to be processed (GAG chain attachment and product secretion) (18). Both of these results may be consistent and offer no conflicts to our finding. It is also important to note that these studies used different motifs (fragments of the CS domain), which may affect the results. For example, a construct containing a CS fragment of aggrecan is poorly synthesized, and the product is poorly secreted (21), whereas a construct containing a versican CS fragment is well synthesized, and the product is well secreted (20).

All of these results point to the central importance of the G3 domain: it facilitates GAG chain attachment and enhances product secretion. In this study, our goal was to determine which function is more important in aggrecan processing. This question has, in the past, proven difficult to address, since the two effects of G3 are always observed in concert in constructs containing a CS fragment and the G3 domain. Thus, our strategy was to search for domain combinations capable of affecting one function but not the other and to examine if the cysteine residues in the CRD and CBP motif have any effect on product secretion and GAG modification.

Cysteine residues form disulfide bonds and play important functions in maintaining the secondary structure of a protein. The positions of the cysteine residues in aggrecan G3 domain of different species are 100% conserved. However, it is not clear if the cysteine residues in the G3 domain and, by extension, the conformation of G3 affect product processing. To test this, we mutated nearly every cysteine residue systematically. We anticipated that mutagenesis of the cysteine residues would alter the conformation of the products, although the exact effect is not clear, since mutagenesis of one cysteine residue may completely alter the formation of disulfide bonds between other pairs of cysteine residues. Our studies demonstrated that removal of the two cysteine residues in the amino fragment of the CRD motif and the cysteine residue in the amino terminus of CBP exerted effects on product secretion of CRD and CBP constructs, respectively. Mutation of these cysteine residues reduced the amount of product processed and secreted. Some of these mutated fragments, CRDC3 and CBPC2, were thus used to examine their effect on GAG modification. The results that the product of CRDC3 and CBPC2 constructs were poorly secreted but the presence of these two fragments allowed GAG chain attachment to G1CSDCRDC3 and G1CSDCBPC2 constructs and facilitates product secretion indicated that the functions of an element in GAG chain modification and product secretion are separable. The results also suggested that the effect of an element on GAG chain modification could override its effect on product secretion. Because CRD and CBP are the major components of the G3 domain, our results imply that the G3 domain may play a similar role. To support this notion, we searched for a fragment that could be secreted on its own but did not facilitate GAG chain modification nor product secretion. Fortunately, although we found that most of the fragments tested, including G3, CRD, CRDCBP, CRDC0, CRDC4, and CBPC1C2, enabled GAG chain modification and product secre-
tion, we found that the product of the CBPtail construct was secreted, but the product of G1CSDCBPtail was not modified by GAG chains, nor could the product of G1CSDCBPtail be secreted. They indicated that the effect of an element on GAG chain modification is more important than its effect on product secretion. The above results suggested that the function of the G3 domain in product secretion may not be required for GAG chain attachment. However, the role of the G3 domain in GAG chain attachment will indirectly (through the positive effect of the GAG chain on product secretion) affect product secretion. The role of the G3 domain in GAG chain attachment may be able to override its effect on product secretion for aggrecan processing. This conclusion is in agreement with recent publications from other laboratories (16–19). Without GAG chain

![Fig. 6. Fragments enhance GAG chain attachment and product secretion](image)

![Fig. 7. Product secretion is the consequence of GAG modification.](image)
G3 in GAG Modification and Secretion

modification, the product cannot be secreted, as in the case of chicken nanomelia (13, 14).

A related function is the role of G3 domain in intracellular trafficking. In particular, before GAG chain attachment, G3 must play a role in the intracellular trafficking of aggrecan core protein. This will determine the fate of a core protein. If a core protein is folded properly, it may be processed through the endoplasmic reticulum and Golgi and undergo GAG chain addition. If a core protein is not properly folded, it will get degraded or retained inside the cell. This is illustrated in nanomelia. As indicated earlier, as a result of a premature stop codon in the aggrecan gene (12), the truncated core protein, lacking the G3 domain, is not modified by GAG attachment and does not get exported (13). It has been demonstrated that the G3 domain plays a role in product trafficking and secretion through interaction with a heat shock protein chaperon (16, 17). Recently, studies have suggested that the folding of G3 domain is involved in product secretion (18) and that this domain plays a role in product stability and intracellular trafficking (19). In our recent publication, we demonstrated that aggrecan has two elements (G1 and G2 domains) that inhibit product secretion. Aggrecan also has two elements (G3 domain and GAG chain addition) that enhance product secretion. The G3 domain also enables GAG chain attachment. Secretion of a recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements. For example, the product of a construct containing recombinant product depends on the balance of these four elements.

In summary, our work has demonstrated a separation in the functions of elements in the G3 domain in GAG chain attachment and product secretion. Using a double mutant of the CRD motif, we have shown that the effect of an element on GAG chain attachment is more important in product processing than its role in product secretion. Furthermore, we have shown that the cysteine residues in G3 are involved in product secretion but that GAG chain attachment is likely independent of molecular conformation in the G3 domain. The effect of the G3 domain can be substituted by the CRD motif, which does not need to be disulfide-bonded. On the other hand, CBP alone seems to be a negative regulator, but this negative effect is eliminated by mutations, which presumably disrupt the formation of disulfide bonds. Further study will be needed to confirm the biological importance of these findings in aggregan processing.

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The Folded Modules of Aggrecan G3 Domain Exert Two Separable Functions in Glycosaminoglycan Modification and Product Secretion
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