N-Linked Keratan Sulfate in the Aggrecan Interglobular Domain Potentiates Aggrecanase Activity*

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Keratan sulfate is thought to influence the cleavage of aggrecan by metalloenzymes. We have therefore produced a recombinant substrate, substituted with keratan sulfate, suitable for the study of aggrecan analysis in vitro. Recombinant human G1-G2 was produced in primary bovine keratocytes using a vaccinia virus expression system. Following purification and digestion with specific hydrolases, fluorophore-assisted carbohydrate electrophoresis was used to confirm the presence of the monosulfated Gal-GlcNAc6S and GlcNAc6S-Gal disaccharides and the disulfated Galβ2-GlcNAc6S disaccharides of keratan sulfate. Negligible amounts of fucose or sialic acid were detected, and the level of unsulfated disaccharides was minimal. Treatment with keratanases reduced the size of the recombinant G1-G2 by ~5 kDa on SDS-PAGE. Treatment with N-glycosidase F also reduced the size of G1-G2 by ~5 kDa and substantially reduced G1-G2 immunoactivity with monoclonal antibody 5-D-4, indicating that keratan sulfate on the recombinant protein is N-linked. Cleavage of G1-G2 by aggrecanase was markedly reduced when keratan sulfate chains were removed by treatment with keratanase, keratanase II, endo-β-galactosidase, or N-glycosidase F. These results indicate that modification of oligosaccharides in the aggrecan interglobular domain with keratan sulfate, most likely as asparagine residue 368, potentiates aggrecanase activity in this part of the core protein.

Aggrecan is a major structural component of cartilage and together with type II collagen it enables this tissue to bear load and resist compression. Aggrecan has three globular domains, G1 and G2 at the N terminus and G3 at the C terminus. An extended sequence between the G2 and G3 domains is heavily substituted with chondroitin sulfate and keratan sulfate chains, organized into distinct chondroitin sulfate-1, chondroitin sulfate-2, and keratan sulfate-rich regions. An interglobular domain (IGD) of ~150 amino acids separates G1 from G2 and is substituted with keratan sulfate chains as well as O-linked and N-linked oligosaccharides.

The IGD is highly sensitive to proteinases. Cleavage in the IGD releases the entire chondroitin sulfate and keratan sulfate-rich regions essential for the biomechanical properties of aggrecan and is therefore the most detrimental for cartilage function. In pathology, proteolysis is driven by aggrecanases and, to a lesser extent, by matrix metalloproteinases. Aggrecanase was first identified as a novel activity that cleaved the aggrecan core protein at the E373 A bond in the IGD (1-3); the products of this cleavage were found in synovial fluids from patients with osteoarthritis, joint injury, and inflammatory joint disease (4, 5). Subsequently, cartilage enzymes with aggrecanase activity were revealed as members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family (6, 7), and ADAMTS5 has recently been identified as the major aggrecanase in mouse cartilage (8, 9).

Proteolysis by matrix metalloproteinases at the N341 F bond in the IGD does not appear to contribute to glycosaminoglycan loss during early phases of experimental arthritis in mice. However, matrix metalloproteinase cleavage at N341 F correlates with late-stage cartilage damage in mouse models of arthritis (10-12), and it may also be involved in the baseline turnover of aggrecan in vitro (13) and in vivo (14). The products of in vitro proteolysis at both the matrix metalloproteinase and the aggrecanase sites have been found in humans (4, 15, 17, 18) and in mice with experimental arthritis (11, 19–21).

Keratan sulfate is a glycosaminoglycan with a β1,3-linked backbone of the repeating disaccharide Galβ1,4GlcNAc. The majority of the GlcNAc residues and a significant proportion of adjacent Gal residues are sulfated in the C6 position (22, 23), giving rise to mono- or disulfated regions, respectively. Keratan sulfate may also be fucosylated at GlcNAc6S within monosulfated chain regions (23–25) and contain sialic acid capping at the nonreducing Gal or Galα6S (26–28).

Bovine aggrecan contains 2–3 N-linked keratan sulfate chains and 20 or more O-linked keratan sulfate chains (29), most of which are present in the keratan sulfate-rich region between the G2 globular domain and the chondroitin sulfate-rich region. Substitution in the keratan sulfate-rich region is O-linked (30), however, keratan sulfate in the IGD is attached

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1 The abbreviations used are: IGD, interglobular domain (of aggrecan); FACE, fluorophore-assisted carbohydrate electrophoresis; HA, hyaluronan; DMEM/F12, Dulbecco’s modified Eagle’s medium/Ham’s F12; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; EβG, endo-β-galactosidase.

This paper is available on line at http://www.jbc.org
via both O- and N-linkages (29). Studies with aggrecan from porcine and bovine cartilage show that keratan sulfate attachment in the IGD is between the matrix metalloproteinase (N\(^{341}\) \(\rightarrow^{344}\)F) and aggrecanase (E\(^{373}\) \(\rightarrow^{374}\)A) cleavage sites, at threonine residues Thr\(^{352}\), Thr\(^{357}\), and Thr\(^{370}\) and asparagine residue Asn\(^{368}\) (29, 31). A number of studies have shown that cleavage at the E\(^{373}\) \(\rightarrow^{374}\)A bond is inhibited by exogenous glycosaminoglycans (32–34) and highly sulfated polymers (35). Other studies comparing the presence or absence of endogenous keratan sulfate chains have suggested that aggrecanase cleavage in the IGD may be increased in the presence of keratan sulfate and reduced when keratan sulfate is enzymatically removed (36, 37). However, recombinant substrates that lack keratan sulfate are not totally resistant to cleavage by aggrecanases (38, 39). Collectively, these studies suggest that keratan sulfate in the IGD may influence aggrecanolyis.

In this study, we report generation of a recombinant G1-G2 bearing N-linked keratan sulfate, and we show that this N-linked substitution is sufficient to potentiate aggrecanase cleavage in the IGD. To our knowledge, this is the first report of a recombinant protein substituted with keratan sulfate.

**MATERIALS AND METHODS**

**Reagents**—Collagenase II was from Worthington. Monoclonal antibody 1-C-6 (40) was from the Developmental Studies Hyridoboma Bank established under the auspices of the National Institute of Child Health and Human Development and maintained at The University of Iowa, Department of Biological Sciences (Iowa City, IA). 1-C-6 recognizes a reduced epitope in the G1 and keratanase-digested G2 domain (40, 41) of aggrecan. DMEM/F12 was from Thermo Trace. Monoclonal antibody 5-D-4, which recognizes a highly sulfated region in keratan sulfate (40, 42), was a gift from Prof. Bruce Caterson (University of Wales, Cardiff, Wales). Endo-\(\beta\)-galactosidase (ESG), keratanase, keratanase II, and chondroitinase from Seikagaku, N-Glycosidase F, and \(\alpha\)-galactosidase were from Roche Diagnostics. F, \(\alpha\)-1,3-4 Fucosidase and monogals for analysis of depolymerized keratan sulfate chains were from Prozyme, distributed by Epitope Technologies (Melb, Australia). The analytical BioSep-Sec-S 4000 size exclusion HPLC column was from Phenomenex. The vaccinia virus encoding T7-polymerase, T7F3-7, has been described previously (43).

**Generation of vG1-G2**—A construct for producing vaccinia virus expressing G1-G2 was made by subcloning cDNA encoding a human G1-G2 fragment of aggrecan (38) into the pTM1 vector (44) at the Sacl and Xhol cleavage sites. The G1-G2 insert was repositioned in the vector so that the initiating ATG codon was part of the Ncol cloning site in the vector. DMEM/F12 containing 10% fetal calf serum and antibiotics was infected with vTF7-3 alone (control) or co-infected with vTF7-3 and vG1-G2 for 6–7 h at 37 °C. The media were replaced with methionine and cysteine-deficient DMEM. \(^{[35S]}\)Methionine (10 \(\mu\)Ci/ml) was added to the wells, and the infection continued at 37 °C for various times up to 4 days. Aliquots of media were precipitated overnight at −20 °C with 3 volumes of ethanol containing 100 mM ammonium acetate, and the precipitate was recovered by centrifugation. The pellet, radiolabeled samples were analyzed by non-reducing SDS-PAGE and fluorography.

**Expression of \(^{[35S]}\)Methionine-labeled G1-G2 in Cell Monolayers**—Confluent cell monolayers cultured in DMEM/F12 with 2% fetal calf serum and antibiotics were infected with vTF7-3 alone (control) or co-infected with vTF7-3 and vG1-G2 for 6 h at 37 °C. The media were replaced with methionine and cysteine-deficient DMEM. \(^{[35S]}\)Methionine (10 \(\mu\)Ci/ml) was added to the wells, and the infection continued at 37 °C for various times up to 4 days. Aliquots of media were precipitated overnight at −20 °C with 3 volumes of ethanol containing 100 mM ammonium acetate, and the precipitate was recovered by centrifugation. The pellet, radiolabeled samples were analyzed by non-reducing SDS-PAGE and fluorography.

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**Glycosidase Digestion**—Glycosidase digests were done at 37 °C for 18–20 h. Keratanase II (50 milliunits/ml) and EGH (50 milliunits/ml) digests were done in 0.1 mM ammonium acetate buffer, pH 6.0. Neuraminidase (27.7 milliunits/ml) and fucosidase (1 milliunit/ml) digests were done in 0.1 mM ammonium acetate buffer, pH 5.0. N-Glycosidase F (50 units/ml) digests were done in 0.125 mM Tris, pH 6.8, 1% SDS, and 0.6% Nonidet P-40 after denaturing the samples by boiling for 5 min. O-Glycosidase (50 milliunits/ml) digests were done in 20 mM sodium phosphate, pH 7.2, 1% SDS, and 0.5% Nonidet P-40 after denaturing the samples by boiling for 5 min.

**Digestion of G1-G2 with Aggrecanase**—Conditioned medium was harvested from explants of pig articular cartilage (2.75 g) cultured for 4 days in the presence of 1 \(\mu\)M retinoic acid and 10 ng/ml interleukin-1 \(\alpha\) in serum-free DMEM. The medium was concentrated 9-fold on Milli- pore 10-kDa cutoff centrifuge filter units and used as a source of aggrecanase activity, as described previously (13). G1-G2 (1–3 \(\mu\)g) was treated with or without glycosidases and then digested with 1 \(\mu\)L of aggrecanase-containing medium at 37 °C for the times given in buffer containing 50 mM Tris, pH 7.5, 0.1 mM NaCl, and 10 mM CaCl\(_2\). The digestion was stopped by the addition of 10 mM EDTA and 2 mM 1,10-phenanthroline to inhibit aggrecanase activity. Equal amounts of G1-G2, with or without glycosidase or aggrecanase treatment, were

strainer to remove undigested tissue debris. The cells were washed once in medium containing 10% fetal calf serum and twice in serum-free medium and then seeded at a density of 1.11 \(\times 10^6\) cells/cm\(^2\) in DMEM/ F12 medium containing 2% fetal calf serum and antibiotics. All experiments were done with non-passaged primary cells.
Fig. 1. Expression of G1-G2 by primary keratocytes. a, keratocytes isolated from fresh bovine corneas were infected with increasing amounts of vTF7-3 and vG1-G2 (lanes 2, 4, 6, 8, and 10) or with vTF7-3 alone (lanes 1, 3, 5, 7, and 9) and incubated in the presence of [35S]methionine for 40 h. Precipitated samples were analyzed by SDS-PAGE and fluorography. b, the fluorograph was analyzed by scanning densitometry. c, confluent monolayers of primary bovine keratocytes (●) or COS-7 cells (□) were co-infected with vG1-G2 and vTF7-3, and aliquots of the medium were harvested every 12 h for 4 days or 2.5 days, respectively. [35S]-labeled G1-G2 was digested by chondroitinase ABC (ChABC; lane 2), E9G (lane 3), keratanase II (KII; lane 4), keratanase (K; lane 5), or undigested (lane 1) and analyzed by SDS-PAGE and fluorography.

RESULTS

Recombinant human G1-G2 was expressed in primary bovine keratocytes, using a vaccinia virus expression system. To determine the viral load required for adequate G1-G2 expression, cells were co-infected with increasing amounts of vTF7-3 and vG1-G2 in the presence of [35S]methionine (Fig. 1a). A single product of the expected size (approximate molecular mass, 120 kDa) was expressed by the co-infected cells (Fig. 1a, lanes 2, 4, 6, 8, and 10) but was absent in the control cells, which were infected with vTF7-3 alone (Fig. 1a, lanes 1, 3, 5, 7, and 9). Five plaque-forming units/cell generated readily detectable amounts of G1-G2, and scanning densitometry showed that the amount of secreted G1-G2 was proportional to the viral load, up to 20 plaque-forming units/cell (Fig. 1b). An inoculation of 5 plaque-forming units/cell vG1-G2 was used for all subsequent experiments. A time course experiment to determine the optimum infection time showed that G1-G2 was detected in the conditioned medium of the primary keratocytes 24 h post-infection, and its expression continued for up to 4 days (Fig. 1c). In contrast, COS-7 cells infected in the same way produced low levels of G1-G2 during the first 60 h but then died, presumably due to the burden of infection.

When [35S]methionine-labeled G1-G2 was digested with keratanase, keratanase II, or E9G, the size of the band was reduced by ~5 kDa (Fig. 1d, lanes 3–5). As expected, digestion with chondroitinase ABC did not change the size of [35S]-labeled G1-G2 (Fig. 1d, lane 2). The results indicate that the amount of keratan sulfate substituted on G1-G2 expressed in keratocytes (~5 kDa) is substantially less than the amount (~30 kDa) substituted on native G1-G2 from pig laryngeal aggrecan.

Purification of G1-G2 by HA-Sepharose and Size Exclusion Chromatography—The high-affinity binding of the aggrecan G1 domain to HA was exploited for the purification of G1-G2.

Fig. 2. Purification of G1-G2 by HA-Sepharose affinity chromatography and HPLC. a, culture medium harvested from infected keratocytes was applied to an HA-Sepharose column equilibrated in PBS and washed with 0.5 M NaCl (lanes 1, 4, and 7) followed by 1.0 M NaCl (lanes 2, 5, and 8). G1-G2 was eluted with 4 M GuHCl (lanes 3, 6, and 9). Aliquots were analyzed by SDS-PAGE and silver staining (lanes 1–3) or Western blotting with 5-D-4 (lanes 4–6) or 1-C-6 (lanes 7–9). b, G1-G2 eluted from HA-Sepharose was further purified by size exclusion HPLC under dissociative conditions. c and d, column fractions were analyzed by SDS-PAGE and Western blotting with 1-C-6 (c) and 5-D-4 (d).
Significant (G2bac; n = 22), vaccinia-infected bovine keratocytes (G1-G2; n = 22), and (c) baculovirus-infected insect cells (G1-G2bac; n = 20) were imaged by rotary shadowing electron microscopy.

Medium harvested from infected keratocytes was applied to HA-Sepharose, and serum proteins were removed in washes with PBS and 0.5 and 1.0 M NaCl (Fig. 2a). G1-G2 was eluted with 4 M GuHCl, and a single band of the expected size was detected by silver stain (Fig. 2a, lane 3) and Western blotting with 5-D-4 (Fig. 2a, lane 6) and 1-C-6 (Fig. 2a, lane 9). Contaminating proteins (approximate molecular mass, 50–65 kDa) eluting with GuHCl were detected by silver stain (Fig. 2a, lane 3) and removed by dissociative size exclusion HPLC (Fig. 2, b–d). Two peaks were eluted between fractions 26 and 33 (Fig. 2b). Immunoreactivity with 5-D-4 was detected in fractions 27–32 (Fig. 2d), and fractions 27–29, which contained the majority of the 1-C-6 immunoreactivity (Fig. 2c), were pooled for further analysis by rotary shadowing and FACE. The second peak in the HPLC trace, at fractions 30–33, was excluded from the G1-G2 pool and was not analyzed further.

Rotary Shadowing Electron Microscopy of G1-G2—Rotary shadowing was used to compare G1-G2 carrying ~5 kDa of keratan sulfate with native pig G1-G2 carrying ~30 kDa keratan sulfate with recombinant G1-G2 lacking keratan sulfate (expressed in a baculovirus expression system; G1-G2bac) (38). The average distance (mean ± S.E.) between the recombinant G1-G2 and G1-G2bac was significantly (Fig. 3a). The Mann-Whitney U test revealed that the difference in length between the recombinant G1-G2 and G1-G2bac was significant (p < 0.05). The results are consistent with previous studies suggesting that keratan sulfate substitution in the IGD may influence the overall length and rigidity of the interglobular domain (38).

FACE Analysis of Keratan Sulfate on G1-G2—FACE analysis was done to confirm that the G1-G2 contained keratan sulfate and to determine the extent of sulfation. The specific Gal-GlcNAc6S and Gal6S-GlcNAc6S disaccharides were detected in keratanase II digests (Fig. 4a, lane 2). The ratio of the monosulfated:disulfated products, averaged from two separate preparations, was 1.00:0.90. The monosulfated disaccharide Gal-GlcNAc6S produced by keratanase II digestion was generated in equimolar yield to the equivalent monosulfated disaccharide GlcNAc6S-Gal produced by EβG digestion. Faint bands migrating at the position of the unsulfated GlcNAc-Gal following EβG digestion were barely visible on gels and were below the limit of quantitation. Similarly, sialic acid bands were faintly visible after digestion with neuraminidase, but they were too low to quantitate. No fucose was generated by fucosidase, suggesting that this modification was absent from G1-G2 (data not shown).

We compared the ratios of keratan sulfate disaccharides on G1-G2 with native G1-G2 purified from pig laryngeal aggrecan. The ratio of monosulfated:disulfated products in keratanase II digests of the native G1-G2 was 1.00:1.14 (Fig. 4b, lane 5).

Unsulfated disaccharides present in EβG digests were weakly visible (Fig. 4b, lane 6) but were below the limit for quantitation. EβG digestion also generated a sialylated tetrasaccharide (Fig. 4b, lane 6). Neuraminidase treatment removed the capping sialic acid from this species and generated free sialic acid and the Gal-GlcNAc6S-Gal trisaccharide (Fig. 4b, lane 7). Overall, the results indicate that the keratan sulfate on G1-G2 expressed in bovine keratocytes is less sulfated than keratan sulfate chains on native pig G1-G2 and that the extent of sialic acid capping is low.

N-Linked Keratan Sulfate Influences Aggrecanase Cleavage—We have shown previously that almost all the keratan sulfate in the IGD is located on a 100-amino acid fragment of the IGD between the major and minor matrix metalloproteinase sites, at N341G and D442L, respectively (51, 52). Barry et al. (29, 31) have identified precise sites of keratan sulfate attachment within this region at Thr352, Thr357, Thr370, and Asn368, clustered near the aggrecanase cleavage site. To determine whether G1-G2 expressed in bovine keratocytes carries both O- and N-linked keratan sulfate, we digested it with N-glycosidase F or O-glycosidase and monitored for loss of 5-D-4 epitope by Western blotting. Digestion with N-glycosidase F reduced 5-D-4 reactivity to faint bands (Fig. 5a, lanes 2 and 4) and decreased the size of G1-G2 by ~5 kDa (Fig. 5a, lane 4). O-Glycosidase treatment of G1-G2 did not decrease the size of G1-G2 or reduce immunoreactivity with 5-D-4 (data not shown). Collectively, these results strongly suggest that keratan sulfate on G1-G2 is N-linked and that threonine residues at positions 352, 357, and 370 are not substituted with keratan sulfate.

This interpretation is further supported by Western blotting of a duplicate gel with antibody 1-C-6 (Fig. 5a, lanes 6–8). The blot confirms that N-glycosidase reduced the size of G1-G2 by ~5 kDa (Fig. 5a, lane 7), whereas treatment with O-glycosidase did not reduce the size of G1-G2 (data not shown). Samples digested with a mixture of keratanases followed by N-glycosidase (Fig. 5a, lane 8) were the same size as samples digested with N-glycosidase alone (Fig. 5a, lane 7), reiterating further that the keratan sulfate on G1-G2 is N-linked. It was interesting that a small amount of 5-D-4 immunoreactivity resisted N-glycosidase treatment in some cases (Fig. 5a, lane 4), but not others (Fig. 5a, lane 2). This is likely to reflect the fact that not only is 5-D-4 a particularly good anti-keratan sulfate antibody, but also that the antigen is polyvalent, so tiny amounts of residual antigen are detected with 5-D-4.

Several studies have shown that aggrecanase cleavage is blocked following deglycosylation of aggrecan with chondroitinase ABC and keratanases (36, 37). We therefore investigated whether N-linked keratan sulfate in the IGD is sufficient to potentiate aggrecanase cleavage. Digestion of G1-G2 with keratanase, keratanase II, and EβG, prior to digestion with aggrecanase, significantly reduced aggrecanase cleavage and generation of the 360 kDa fragment (Fig. 5b). These results were reproducible in 9 of 11 experiments and suggested either that G1-G2 without keratan sulfate is a poor substrate for aggrecanase, as reported previously, or, alternatively, that G1-G2 without keratan sulfate is a poor antigen for the anti-NITEGE antibody. To resolve this issue, we compared duplicate Western blots developed with anti-NITEGE or with anti-5-D-4 antibody reactivity. Digestion with N-glycosidase F prior to...
N-Linked Keratan Sulfate Potentiates Aggrecanase Activity

The importance of glycosylation in aggrecanolysis is an emerging theme, initiated by studies showing that deglycosylation of aggrecan inhibits cleavage at aggrecanase sites and suggesting that glycosaminoglycans are required for aggrecanase activity (36, 37). On the other hand, exogenous glycosaminoglycans and highly sulfated polymers inhibit aggrecanase activity (32–35), possibly by interactions with thrombospondin type I motifs (37, 55) or cysteine-rich and spacer domains (54) in ADAMTS enzymes. This anomaly suggests that there may be competition between endogenous and exogenous glycosaminoglycans for binding to ADAMTS enzymes and further implies a direct effect of glycosaminoglycans on aggrecanolysis. Previous studies with keratan sulfate-deficient G1-G2bac suggest that keratan sulfate may also influence aggrecanase activity, but by indirect effects. The mechanism by which keratan sulfate directly or indirectly modulates metalloproteinase activity has not been described, nor have the details of the keratan sulfate microstructure that might be involved in enzyme modulation.

Previously, we have done fragmentation studies analyzing regions of the IGD containing 32 amino acids (Phe342—Glu373), 100 amino acids (Phe342—Asp441), and IGD with the full G2 domain (—110 kDa) (51, 52). Based on size analysis, before and after keratanase treatment, these studies indicate that almost all the keratan sulfate in the IGD is localized on the 32-mer fragment, in the region DETVQTVTWPDMEPLPRNITEGE373 that contains the four keratan sulfate substitution sites (underlined) identified by Barry et al. (29). Pratta et al. (36) have shown that removal of keratan sulfate from purified bovine aggrecan blocks cleavage at E373 (51, 52). These results indicate that N-linked keratan sulfate is sufficient to potentiate aggrecanase cleavage in the IGD.

**Discussion**

To our knowledge, there is no evidence that aggrecan from human articular cartilage is ever found without keratan sulfate. However, the structure of keratan sulfate on human aggrecan changes dramatically with age (55). In contrast with aggrecan from young articular cartilage (0–9 years), aggrecan from adult articular cartilage (18–85 years) is substantially more sulfated and more highly modified by fucosylation and sialylation. Maturing cartilage (9–18 years) has intermediate and increasing levels of sulfation, fucosylation, and sialylation (55). It is interesting to speculate that poten-
tial interactions of ADAMTS thrombospondin type I motifs (37, 53) or cysteine-rich and spacer domains (54) with keratan sulfate may increase with increasing levels of sulfation and chain modification.

It is important to note that the studies on age-dependent changes of keratan sulfate described above were done with keratan sulfate from the keratan-sulfate rich region of aggrecan. We have shown recently, using aggrecan extracted from skeletally mature pig articular cartilage, that keratan sulfate in the keratan-sulfate rich region is not at all similar to keratan sulfate isolated from the IGD (56). Additional studies are required to determine whether keratan sulfate in the IGD changes with age, and, if so, whether those changes correlate with susceptibility to joint disease.

This study is the first to report keratan sulfate substitution on a recombinant protein. The amount of keratan sulfate on pig laryngeal G1-G2 that can be removed by keratanase treatment is ~30 kDa. In contrast, only 5 kDa keratan sulfate was present on G1-G2. This may reflect synthesis of shorter chains or a selective loss of substitution at O-linked sites because keratocytes have not been reported to produce O-linked keratan sulfate.

Unlike most cells in culture, primary bovine keratocytes continue keratan sulfate synthesis in vitro, albeit at reduced levels and with less sulfation (57, 58). The presence of mono- and disulfated disaccharides on G1-G2 indicates that the cells have the enzymes required for polyolactosaminoclan synthesis, as well as the sulfotransferases required for keratan sulfate biosynthesis. This finding is consistent with studies by Funderburgh et al. (59), who detected mono- and disulfated keratan sulfate on endogenous proteoglycans synthesized by cultured bovine keratocytes.

One feature of in vitro keratan sulfate biosynthesis appears to be down-regulation of GlcNAc6ST (60), the sulfotransferase responsible for the sulfation of GlcNAc residues. Because GlcNAc6ST activity occurs simultaneously with chain elongation (61, 62), down-regulation of GlcNAc6ST results in shorter chains with unsulfated disaccharides. In the context of keratan sulfate proteoglycans made by keratocytes in vitro, the G1-G2 reported here is unusual in that it has negligible amounts of unsulfated disaccharides, suggesting that GlcNAc6ST activity is not limiting in this system. Sulfation of galactose by KS-GalNAc6ST (63) and/or C6ST (64) does not appear to be limiting either because ~40% of the disaccharides were sulfated on galactose moieties. Thus, deficiencies in sulfotransferase activities do not appear to account for the reduced level of keratan sulfate on G1-G2. Approximately 60% of the pig laryngeal keratan sulfate was disulfated, compared with 40% for the G1-G2, which may reflect the phenotype of the keratocytes. Similarly, the minimal sialic acid capping and the lack of fucosylation may reflect the keratocyte phenotype because core neuraminic acid sulfate is not fucosylated, and it may be capped with structures other than sialic acid, such as βGalNAc or αGal (16, 65).

In summary, we have produced and characterized a recombinant G1-G2 fragment that is substituted with keratan sulfate. Compared with keratan sulfate in the IGD of pig laryngeal aggrecan, keratan sulfate in the IGD of recombinant G1-G2 is less sulfated, has minimal sialic acid capping, and is exclusively N-linked to the core protein. Removal of the N-linked keratan sulfate diminishes aggrecanase cleavage at the E372 [374] A bond. The data indicate that N-linked keratan sulfate in the aggrecan IGD, most likely at position Asn308, confers susceptibility to aggrecanases.

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