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Lectin affinity chromatography of articular cartilage fibromodulin: Some molecules have keratan sulphate chains exclusively capped by α(2-3)-linked sialic acid

Robert M. Lauder · Thomas N. Huckerby · Ian A. Nieduszynski

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Abstract Fibromodulin from bovine articular cartilage has been subjected to lectin affinity chromatography by *Sambucus nigra* lectin which binds α(2-6)- linked *N*-acetylneuraminic acid, and the structure of the keratan sulphate in the binding and non-binding fractions examined by keratanase II digestion and subsequent high pH anion exchange chromatography. It has been confirmed that the keratan sulphate chains attached to fibromodulin isolated from bovine articular cartilage may have the chain terminating *N*-acetylneuraminic acid residue α(2-3)- or α(2-6)-linked to the adjacent galactose residue. Although the abundance of α(2-6)-linked *N*-acetylneuraminic acid (*ca*. 22%) is such that this could cap one of the four chains in almost all fibromodulin molecules, it was found that *ca*. 34% of the fibromodulin proteoglycan molecules from bovine articular cartilage were capped exclusively with α(2-3)-linked *N*-acetylneuraminic acid. The remainder of the fibromodulin proteoglycans, which bound to the lectin had a mixture of α(2-3)- and α(2-6)-linked *N*-acetylneuraminic acid capping structures. The keratan sulphates attached to fibromodulin molecules capped exclusively with α(2-3)-linked *N*-acetylneuraminic acid were found to have a higher level of galactose sulphation than those from fibromodulin with both α(2-3)- and α(2-6)-linked *N*-acetylneuraminic acid caps, which bound to the *Sambucus nigra* lectin. In addition, both pools contained chains of similar length (*ca*. 8–9 disaccharides). Both also contained α(1-3)-linked fucose, showing that this feature does not co-distribute with α(2-6)-linked *N*-acetylneuraminic acid, although these two features are present only in mature articular cartilage. These data show that there are discrete populations of fibromodulin within articular cartilage, which may have differing impacts upon tissue processes.

Keywords Fibromodulin · Keratan sulphate · *Sambucus nigra* · N-acetylneuraminic acid

Abbreviations

KS	keratan sulphate
GlcNAc/-ol	*N*-acetylglucosamine/*N*-acetylglucosaminitol (2-acetamido-D-glucitol)
NeuAc	*N*-acetylneuraminic acid
6S/(6S)	O-ester sulphate group on C6 present/ sometimes present
Gal A	galactose residue adjacent to the non-reducing terminal *N*-acetylneuraminic acid of the KS chain
Gal B	galactose residue one removed from Gal A
Gal C	any galactose residue in the repeat region of the KS chain
HPAEC	high pH anion exchange chromatography
SNA	S*ambucus nigra* agglutinin
BAC	bovine articular cartilage

Introduction

Fibromodulin is a member of the small leucine-rich proteoglycan (SLRP) family within which five classes have been described [1]. Class I includes the chondroitin/dermatan sulphate (CS/DS) substituted decorin and biglycan [2], while class II contains the keratan sulphate (KS)

R. M. Lauder (✉) · T. N. Huckerby · I. A. Nieduszynski
Faculty of Health and Medicine, Division of Biomedical and Life Sciences, Lancaster University,
Bailrigg,
Lancaster, UK
e-mail: r.lauder@lancaster.ac.uk

substituted lumican [3–5], PRELP [6], keratocan [7], osteoadherin [8] as well as fibromodulin [9]. Fibromodulin, first isolated from bovine articular cartilage [9], and subsequently cloned and sequenced from bovine tracheal cartilage [10], has a tyrosine-rich domain at its N-terminus, in which some of the tyrosine residues are sulphated [11] and which is susceptible to cleavage by MMP-13 [12] but which is also reported to bind MMP-13 along with other factors including basic fibroblast growth factor-2 [13].

It is exclusively glycosylated by short chains of KS [14–18] and Plaas *et al*., [19] have demonstrated that, in fibromodulin from 3-month-old bovine articular cartilage, only four out of the five potential glycosylation sites are substituted by either KS or an N-linked oligosaccharide.

Keratan sulphates have been classified according to their mode of linkage to amino acids as KS-I for N-linked chains, and KS-II for O-linked chains [20]. A third type, O-linked from mannose to serine or threonine, has been isolated from brain tissue [21]. Keratan sulphate, which is based upon a repeating *N*-acetyllactosamine sequence of -4GlcNAcß1-3Galß1-, is usually sulphated on C-6 of *N*-acetylglucosamine (GlcNAc), and further sulphate groups may be present on C-6 of galactose (Gal) [22–26].

Nieduszynski *et al*., [27, 28] and Lauder *et al*., [14, 16, 18] have demonstrated that, for O- and N-linked KS chains, respectively, there is a distinction between chains from articular cartilage or cornea, which can contain (α2-6)-linked *N*-acetylneuraminic acid (NeuAc) chain caps and (α1-3)-linked fucose, and chains from other tissues which contain neither of these features [14]. Lauder *et al*., [18] and Brown *et al*., [29] have shown that the abundance of these structures is age dependent, being absent in immature articular cartilage but present in that from older tissue.

There is evidence that fibromodulin, along with decorin, has a central role in the maintenance of the structure of the collagen fibrils, which make up the extracellular matrix of cartilage. Fibromodulin has been found to inhibit the formation of collagen fibrils *in vitro* [30, 31], and binds to collagens I and II [31–34] where it may inhibit degradation [35].

The core protein of fibromodulin binds to collagen monomers, but it is the attached KS chains, which apparently control fibril diameter and interfibrillar spacing [36, 37]. Studies have shown that the conformation of terminal NeuAc residues varies significantly with the linkage type [38]. Thus, changes in the length of the KS chains attached to fibromodulin, or in the incidence of specific structures such as chain caps, may affect the structure of developing collagen fibrils. It is noteworthy that fibromodulin null mice suffer increased levels of osteoarthritis [39], although the reason for this is not established.

Keratan sulphate is a complex polymer and analysis of its structure while intact is challenging [24–26], and so analysis is often performed upon the products of depolymerisation [14–18]. Keratanase II, an endo-β-*N*-acetylglucosaminidase, cleaves the β(1–3)-glycosidic bond of a 6-O-sulphated GlcNAc in KS [40] and it has been useful when combined with high pH anion exchange chromatography (HPAEC) [14–18, 41, 42] or NMR spectroscopy [15] for KS structure analysis.

The elderberry derived lectin *Sambucus nigra* agglutinin (SNA) has been shown to bind α(2-6)-linked NeuAc strongly whilst having only a low affinity for α(2-3)-linked NeuAc [43–46]. The biological significance of α(2-6)-linked NeuAc is seen in work, which has demonstrated it to be important in renal function [47], be a marker for aggressive malignancy in colorectal carcinoma [48, 49] undergo changes in expression in oral carcinogenesis [50] and have an involvement in malaria [51]. However, its role in the KS chains of articular cartilage fibromodulin is not clear.

Previous work examined the structure of KS chains from bovine articular cartilage aggrecan following their separation into two populations based upon SNA binding, and found they shared many characteristics [46]. In this study fibromodulin has been isolated from bovine articular cartilage, and the binding of the intact proteoglycan to SNA has been examined. The structures of the KS chains from binding and non-binding populations of fibromodulin have been examined by keratanase II digestion and subsequent analysis by HPAEC.

Experimental

Materials

A Mono-Q 10/10 column and S-300 column media were purchased from GE Healthcare UK Ltd (Little Chalfont, Bucks. UK.), a Brownlee C4 column was purchased from Altech Associates (Carnforth, Lancs. UK) immobilised SNA gel (SNA-agarose) was purchased from EY Laboratories (via Bradsure Biologicals, Market Harborough, Leics. UK). Keratanase II was from Associates of Cape Cod (Deacon Park, UK) and ethylenediamine was from Sigma Chemical Co. (Poole, Dorset. UK). The antibody to the protein core of fibromodulin used in this study was a kind gift of Dr A. H. K. Plaas (Rush University Medical Center, Chicago, USA).

Isolation of fibromodulin from bovine articular cartilage

Fibromodulin was isolated from bovine articular cartilage as previously described [14–18]. Briefly, diced bovine articular cartilage was extracted into 4 M guanidine hydrochloride in the presence of a protease inhibitor cocktail (50 mM sodium acetate, 100 mM 6-aminocaproic acid, 10 mM EDTA, 5 mM benzamidine hydrochloride pH 6.8). The extract was converted to associative conditions by dialysis against the protease inhibitor cocktail, and solid CsCl added to achieve

a density of 1.48 g/ml. Following density gradient centrifugation at 100,000 g for 48 h at 4°C, the A3 & A4 fractions ($\rho<1.53$) were pooled and exchanged into 6 M urea/0.15 M NaCl/50 mM TRIS/HCl pH 7.3 by dialysis.

This material was subjected to ion exchange chromatography at 2 ml/min on a Mono-Q HR 10/10 column, which had previously been equilibrated with 6 M urea/0.15 M NaCl/50 mM TRIS/HCl pH 7.3, bound material being eluted with a linear gradient of 0.15 M – 1 M NaCl in 6 M urea/50 mM TRIS/HCl pH 7.3. The absorbance of the eluate was monitored on-line at 280 nm and column fractions were examined for glycosaminoglycans using the 1,9-dimethylmethylene blue (Taylors Blue) assay [52] and for fibromodulin by an ELISA using an antibody to the protein core [14].

Fibromodulin-containing fractions were pooled, exchanged into 4 M guanidine/50 mM TRIS/HCl pH 7.3, and subjected to size exclusion chromatography on an S-300 column (50 cm×1 cm) eluted in 4 M guanidine/50 mM TRIS/HCl pH 7.3, at 9 ml/h [14–18]. Fibromodulin-containing fractions, identified by ELISA as described above, were pooled and dialysed overnight against 0.2 M NaCl and then extensively against water.

Fibromodulin purity was confirmed by reverse phase chromatography on a Brownlee C4 column; a single component, giving a positive response to 1,9-dimethylmethylene blue [52] and fibromodulin ELISA, eluted at *ca*. 32 min, the position previously reported for fibromodulin [14].

Sambucus nigra agglutinin affinity chromatography

Sambucus nigra agglutinin affinity chromatography was performed as described previously [46]. Briefly, bovine articular cartilage fibromodulin (5 mg), in 1 ml PBS, was applied to a column (8.5 cm×1 cm) of SNA, at 4°C, which had been previously equilibrated with PBS. Non-binding material was eluted by PBS at a flow rate of 0.2 ml/min, and bound material by 20 mM unbuffered ethylenediamine (EDA) at a flow rate of 0.2 ml/min. Fractions were collected and assayed for glycosaminoglycans using the 1,9-dimethylmethylene blue assay [52] and for fibromdulin by an ELISA using an antibody to the protein core [14].

Keratanase II digestion

The KS attached to fibromodulin from binding and non-binding pools was subjected to digestion by keratanase II (0.2 mU/mg of fibromodulin) in 10 mM sodium acetate pH 6.8 for 24 h at 37°C as previously described [15].

Isolation of oligosaccharides

The oligosaccharides generated by keratanase II digestion were isolated as previously described [14–18]. Briefly, following digestion they were reduced by addition of $NaBH_4$ to 1 M. After 3 h at room temperature reduction was stopped by dropwise addition of acetic acid. The reduced oligosaccharides were desalted by chromatography on a column of Bio-Gel P-2 (11 cm×1 cm) eluted in water at 12 ml/h, then lyophilised.

High pH anion exchange chromatography

The composition of the reduced oligosaccharides was examined by HPAEC on a Dionex IonPac AS4A-SC column (250 mm×4 mm), with an AG4A-SC guard column (50 mm×4 mm), maintained at 30°C and eluted at 1 ml/min as described previously [14–18, 53]. An aliquot of reduced oligosaccharides was suspended in water (20 μl) and applied to the column. Elution was by a 5 min isocratic period of 5% 1 M NaOH/95% water, followed by a linear gradient of 0–95% 1.5 M sodium acetate and constant 5% NaOH over 50 min. The eluant was monitored on-line by a pulsed electrochemical detector, configured for integrated pulsed amperometry, using programme 1 (carbohydrates).

Previous work [53] has determined response factors for each oligosaccharide generated by keratanase II digestion of KS and these values were used to determine the relative molar abundance of each oligosaccharide as previously described [15]. The level of fucosylation and Gal sulphation along with the chain length and attachment of NeuAc reported in Fig. 3 were subsequently determined as previously described [15]. Briefly, fucosylation was estimated by determining the percentage of GlcNAc residues having a fucose residue attached; the level of sulphation at Gal C (for codes see Table 1 and Fig. 3) was estimated by determining the percentage of repeat region galactose residues, those within R coded oligosaccharides, which were sulphated; the sulphation level at Gal B was estimated by determining the total abundance of oligosaccharides C2a, C2b and C3 as a percentage of all capping oligosaccharides and the sulphation level at Gal A was estimated by determining the total abundance of C3 as a percentage of all capping oligosaccharides.

The average length of the antennae of KS attached to each sample of fibromodulin was estimated by calculating the ratio of capping oligosaccharides to repeat region oligosaccharides as previously described [15].

Results and discussion

HPAEC analysis of KS

The results of analysis of a sample of KS are shown in Fig. 1. Here the elution of the KS oligosaccharides known to be present in articular cartilage FM [14–18] is seen with

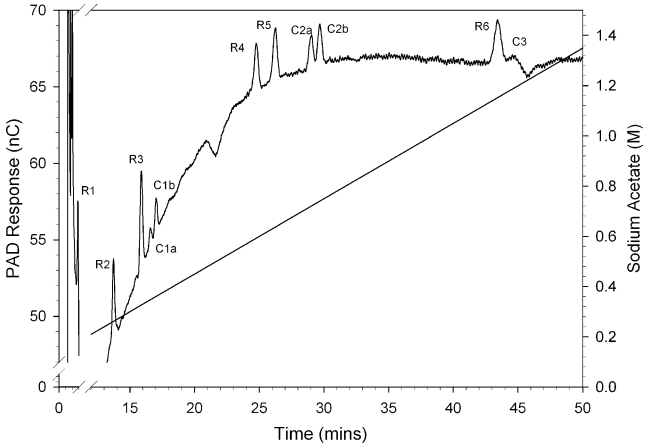

Fig. 1 HPAEC analysis of keratan sulphate. Oligosaccharide composition was examined by HPAEC on a Dionex IonPac AS4A-SC column (250 mm×4 mm), with an AG4A-SC guard column (50 mm×4 mm), maintained at 30°C and eluted at 1 ml/min as described previously [14–18, 53]. Elution was by a 5 min isocratic period of 5% 1 M NaOH/95% water, followed by a linear gradient of 0-95% 1.5 M sodium acetate and constant 5% NaOH over 50 min. The eluant was monitored on-line by a pulsed electrochemical detector, configured for integrated pulsed amperometry, using programme 1 (carbohydrates)

codes as listed in Table 1. Although the estimation of the relative molar abundance of each oligosaccharide requires the application of a response factor [53], it is, for example, possible to see that in this sample the chain caps with one sulphated Gal residue, C2a and C2b, are more abundant than those with no Gal sulphates, C1a and C1b. The rising baseline, reaching a plateau at *ca*. 25 min, is a feature of this method [15].

Sambucus nigra agglutinin affinity chromatography

The results of SNA affinity chromatography of intact bovine articular cartilage fibromodulin are shown in Fig. 2. Three peaks containing material absorbing at 280 nm were identified; of these the first (A) was found to contain no sulphated glycosaminoglycans or material reacting with the anti-fibromodulin antibody. The second and third peaks, B and C, which represented 34% and 66% of the total fibromodulin containing peak areas respectively, as determined by absorbance at 280 nm, were found to react with the anti-fibromodulin antibody and contained sulphated glycosaminoglycans.

The fractions comprising peaks B and C were separately pooled and recovered. To ensure that the non-binding material of peak B contained only material, which would not bind to the column and was not the result of column overloading, an aliquot was rechromatographed as above and was found not to bind to the column (data not shown).

The KS attached to the fibromodulin in each peak was examined by keratanase II digestion and subsequent analysis by HPAEC. The KS attached to fibromodulin from a variety of sources has been well characterised by this technique [14–18] and the expected range of repeat region and capping oligosaccharides were detected. However, the distribution of these oligosaccharides differed between the two populations of fibromodulin.

The KS chains attached to the fibromodulin, which did not bind to the lectin, were found to be capped exclusively by α(2-3) linked NeuAc (Fig. 2). That, which was attached

Table 1 Keratan sulphate oligosaccharides and associated codes

Code	Oligosaccharide
RF1	Galβ1-4(Fucα1-3)GlcNAc(6*S*)
R1	Galβ1-4GlcNAc(6*S*)
R3	Gal(6*S*)β1-4GlcNAc(6*S*)
RF2	Galβ1-4(Fucα1-3)GlcNAc(6*S*)β1-3Galβ1-4(Fucα1-3)GlcNAc(6*S*)
RF3	Galβ1-4GlcNAc(6*S*)β1-3Galβ1-4(Fucα1-3)GlcNAc(6*S*)
RF4	Galβ1-4(Fucα1-3)GlcNAc(6*S*)β1-3Galβ1-4GlcNAc(6*S*)
R2	Galβ1-4GlcNAc(6*S*)β1-3Galβ1-4GlcNAc(6*S*)
RF5	Gal(6*S*)β1-4GlcNAc(6*S*)β1-3Galβ1-4(Fucα1-3)GlcNAc(6*S*)
RF6	Galβ1-4(Fucα1-3)GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)
R4	Gal(6*S*)β1-4GlcNAc(6*S*)β1-3Galβ1-4GlcNAc(6*S*)
R5	Galβ1-4GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)
R6	Gal(6*S*)β1-4GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)
CF1	NeuAcα2-3Galβ1-4GlcNAc(6*S*)β1-3Galβ1-4(Fucα1-3)GlcNAc(6*S*)
C1a	NeuAcα2-6Galβ1-4GlcNAc(6*S*)β1-3Galβ1-4GlcNAc(6*S*)
C1b	NeuAcα2-3Galβ1-4GlcNAc(6*S*)β1-3Galβ1-4GlcNAc(6*S*)
C2a	NeuAcα2-6Galβ1-4GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)
C2b	NeuAcα2-3Galβ1-4GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)
C3	NeuAcα2-3Gal(6*S*)β1-4GlcNAc(6*S*)β1-3Gal(6*S*)β1-4GlcNAc(6*S*)

Oligosaccharides known to be generated by keratanse II digestion of keratan sulphate are listed along with their associated codes as previously reported [14–18, 53]

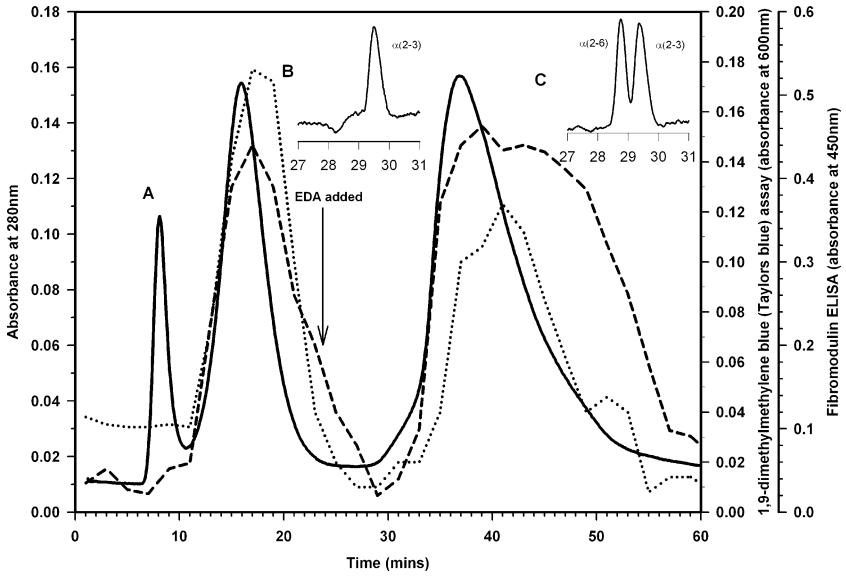

Fig. 2 SNA affinity chromatography of bovine articular cartilage fibromodulin. A sample of fibromodulin, in PBS, was applied to the SNA column (8.5 cm×1 cm); which had been previously equilibrated with PBS. Non-binding material was eluted by PBS at a flow rate of 0.2 ml/min., and binding material by 0.1% EDA at a flow rate of 0.2 ml/min. The absorbance of the eluant was monitored at 280 nm (——) and the fractions were assayed for sulphated glycosaminoglycans by the 1,9-dimethylmethylene blue (Taylors Blue) assay [52] (– – – –) and for fibromodulin by an ELISA [14] (·····). A 20 μl aliquot of reduced oligosaccharides derived by keratanase II digestion of the KS attached to each population of fibromodulin was suspended in water and applied to an Dionex AS4A-SC column. The eluent was monitored using on-line by a pulsed electrochemical detector, configured for integrated pulsed amperometry, using programme 1 (carbohydrates). A 5 min isocratic period of 5% 1 M NaOH/95% water, was followed by a linear gradient of 0-95% 1.5 M sodium acetate and constant 5% NaOH over 50 min. A short portion of the chromatogram is shown, within which examples of both NeuAc caps elute

to the fibromodulin, which bound to the lectin was found to contain both α(2-6)- and α(2-3)-linked caps.

In fibromodulin, which bound to SNA, α(2-6) linked NeuAc caps were found to terminate 34% of the KS chains while in the total sample α(2-6)-linked NeuAc caps were found to terminate 22% of the chains, a value in agreement with our previous studies of the incidence of capping features of KS from articular cartilage fibromodulin [14–18] and aggrecan [46].

The SNA lectin shows very tight binding of α(2-6)-linked NeuAc, and a single α(2-6)-linked NeuAc chain cap is sufficient for binding. Previous work has utilised this property to isolate chains capped by α(2-6)-linked NeuAc [46]. These observations are consistent with our data which show that no fibromodulin carrying an α(2-6)-linked NeuAc chain cap failed to bind to the SNA.

That fibromodulin, which did not bind to the SNA should contain no α(2-6)-linked NeuAc is not surprising as the lectin specifically binds such a structure. The important observation from these data is that in adult bovine articular cartilage *ca*. 34% of fibromodulin molecules contain no α(2-6)-linked NeuAc chain caps.

Previous work has shown that α(2-6)-linked NeuAc chain capping of KS in articular cartilage is found only in mature tissues [14–18]. Thus, α(2-6)-linked NeuAc caps, which account for 22% of the caps in this tissue, are not distributed equally amongst fibromodulin molecules, some of which in mature articular cartilage lack a feature normally associated with that tissue [18].

Fucosylation

Several fucosylated oligosaccharides have been observed in KS [14–18] (Table 1). Of these the di-fucosylated RF2 and fucosylated chain cap CF1 were not observed in either the population of fibromodulin, which bound to the SNA lectin, or that which did not. Each of the other fucoslyated oligosaccharides RF1, RF3, RF4, RF5 and RF6 were observed in both populations. The relative abundance of these fucosylated oligosaccharides was similar, in both SNA binding and non-binding populations, to that previously reported for fucosylated fibromodulin [14–18].

The level of GlcNAc fucoslyation in binding and non-binding pools of fibromodulin was similar *ca*. 3.1 - 3.4% (Fig. 3). This is lower than the levels previously observed for KS from bovine articular cartilage fibromodulin [18], and highlights the variability in the abundance already noted for this residue [18]. These data show that fucose, only found in mature articular cartilage fibromodulin, is not restricted to chains capped by α(2-6)-linked NeuAc, which is also only found in mature articular cartilage. It has also been shown that α(2-6)-linked NeuAc and α(1-3)-linked

fucose are not co-distributed in KS from articular cartilage aggrecan [46].

Chain length

The average length of the cap and repeat region of the KS chains attached to fibromodulin, which did not bind to the lectin is *ca*. 9 disaccharides, that from fibromodulin which did bind to the lectin is *ca*. 8 disaccharides (Fig. 3). These data are in close agreement with our previous work examining the structure of the KS attached to fibromodulin from mature cartilage, in which it was found that the length of the repeat region and antennar cap were *ca*. 8–9 disaccharides in human [14–18], equine [14–18] and bovine articular cartilage [18] and *ca*. 5–7 disaccharides from bovine tracheal cartilage [15].

This suggests that chains capped by α(2-6)-linked NeuAc are not significantly different in size from those capped by α(2-3)-linked NeuAc. Further, no age related differences were reported in the overall average chain length of KS [18] despite an increase in the abundance of α(2-6)-linked NeuAc. The length of KS chains attached to aggrecan has also been shown to be independent of chain cap [46].

Galactose sulphation

A range of fucosylated and non-fucosylated repeat region oligosaccharides have been observed in KS [14–18] (Table 1). Fucosylation, known to be associated with unsulphated Gal residues [14–18], was found on a small percentage of the total repeat region oligosaccharides (Fig. 3). All of the un-fucosylated oligosaccharides previously reported for fibromodulin [14–18] were detected in both SNA binding and non-binding populations. The relative abundance of these repeat region oligosaccharides was similar, in both populations, to that previously reported for fibromodulin [14–18].

The HPAEC data demonstrate that the Gal residue adjacent to the terminal NeuAc (Gal A) is sulphated in *ca*. 2.3–2.4% of the chains (Fig. 3) irrespective of the binding status, however, this residue may only be sulphated when it is involved in an α(2-3)-linkage to NeuAc as the C6 position is otherwise occupied by NeuAc, and not available for sulphation. Thus, in the binding pool if only chains which terminate in α(2-3)-linked NeuAc are considered then the level of sulphation of this penultimate residue where the position is free for sulphation increases to *ca*. 3.5%.

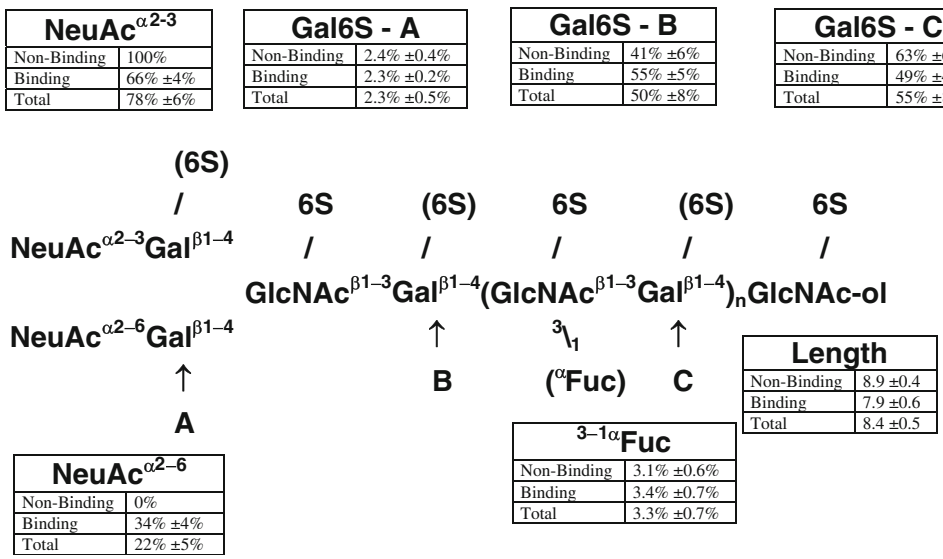

Fig. 3 General structure of the N-linked keratan sulphate attached to articular cartilage fibromodulin. The general structure of articular cartilage fibromodulin KS is shown with the incidence of various features found in the total population of fibromodulin (Total), the population of fibromodulin, which bound to the SNA lectin (Binding) and the population, which did not bind to the SNA lectin (Non-binding). The galactose residues referred to as Gal A, Gal B and Gal C are indicated. Fucosylation (Fuc) represents the percentage of GlcNAc residues having a fucose residue attached; Gal6s C represents the percentage of sulphated repeat region galactose residues, Gal6S B is an estimate of the abundance of oligosaccharides C2a, C2b and C3 as a percentage of all capping oligosaccharides and Gal6S A is an estimate of the total abundance of C3 as a percentage of all capping oligosaccharides. The average number of disaccharides isolated per NeuAc residue was determined by examination of the ratio of the abundance of repeat region oligosaccharides to capping oligosaccharides. Each capping oligosaccharide contributes two disaccharides, as does each repeat region tetrasaccharide and their fucosylated variants. Therefore, the average number of disaccharides (−3Galß1-4GlcNAcß1-)$_n$ isolated per NeuAc residue is calculated by: n = (Disaccharides) + (Tetrasaccharides x 2) + (Pentasaccharide[c] x 2)/ Pentasaccharide caps. [a] Disaccharides and fucosylated trisaccharide. [b] Tetrasaccharides and fucosylated penta and hexasaccharides. [c] Pentasaccharide and fucosylated hexasaccharide caps

These data are in agreement with the levels of sulphation of this important sub-terminal residue in mature bovine tracheal [14, 15] and articular [16] cartilage fibromodulin. However, very much higher levels of sulphation of this residue (*ca*. 30%) have been observed in fibromodulin from mature human and equine articular cartilage [16–18].

The level of sulphation of the Gal reside one removed from Gal A (Gal B) is much higher, with binding chains having a higher level of sulphation (*ca*. 55%) than non-binding (*ca*. 41%). The average sulphation level of this Gal residue in the total KS pool (*ca*. 50%) agrees with our previous data of 50% and 58% from bovine tracheal and articular cartilage respectively [14–16], and 49% from equine articular cartilage [16].

High levels of repeat region sulphation (Gal C) were observed for the binding (49%) and non-binding pools (63%), suggesting that chains capped by α(2-3)-linked NeuAc may be more highly sulphated within their repeat regions than those capped by α(2-6)-linked NeuAc. This possibility is supported by previous data demonstrating up to 60% Gal sulphation in bovine tracheal cartilage fibromodulin [14–18], which is exclusively capped by α(2-3)-linked NeuAc. However, previous data examining age related changes in KS structure detected no change in sulphation with increasing α(2-6)-linked NeuAc abundance [14–18].

General discussion

In this work we have isolated a pool of fibromodulin from mature bovine articular cartilage which is exclusively capped by α(2-3)-linked NeuAc and a second pool capped by α(2-3)- and α(2-6)-linked NeuAc. The KS chains from these two pools are similar in length and fucosylation, although there was a higher level of Gal sulphation in the KS from fibromodulin, which did not bind to the SNA lectin.

Previously Tai *et al*. [46] examined the KS chains attached to bovine articular cartilage aggrecan and showed that the α(2-6)-linked NeuAc chain cap represents around 30% of the total caps, that this chain cap and α(1-3)-linked fucose do not co-distribute and that the chain sizes of the chains, which did bind to the SNA lectin and those, which did not bind to the SNA lectin, did not differ. We have shown that these outcomes are also true for fibromodulin despite the different KS linkages of these two proteoglycans.

These data do not tell us if the KS chains attached at each of the potential KS attachment sites in fibromodulin may each be capped by either α(2-3)- or α(2-6)-linked NeuAc. Studies of aggrecan show that the KS attached to different sites within the core protein have different structures [54].

These data extend our understanding of the KS of articular cartilage, showing similarities between the KS on different proteoglycans and attached with different linkage types. These data focus attention to the chain caps as an important site for the modulation of structure. It is possible that an α(2-6)-linked NeuAc chain cap may confer properties relating either to the chain's resistance to degradation or to specific recognition phenomena. Further, it is known that the α(2-6)-linked NeuAc cap has a significantly different conformation to that adopted by α(2-3)-linked NeuAc [38]. Therefore, it is possible that chains capped by α(2-6)-linked NeuAc may have an altered ability to influence fibril diameter and interfibrillar spacing.

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