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Chondroitin sulfate and keratan sulfate are the major glycosaminoglycans present in the adult zebrafish *Danio rerio* (Chordata-Cyprinidae)

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Abstract The zebrafish *Danio rerio* (Chordata-Cyprinidae) is a model organism frequently used to study the functions of proteoglycans and their glycosaminoglycan (GAG) chains. Although several studies clearly demonstrate the participation of these polymers in different biological and cellular events that take place during embryonic development, little is known about the GAGs in adult zebrafish. In the present study, the total GAGs were extracted from the whole fish by proteolytic digestion, purified by anion-exchange chromatography and characterized by electrophoresis after degradation with specific enzymes and/or by high-performance liquid chromatography (HPLC) analysis of the disaccharides. Two GAGs were identified: a low-molecular-weight chondroitin sulfate (CS) and keratan sulfate (KS), corresponding to ~80% and 20% of the total GAGs, respectively. In the fish eye, KS represents ~ 80% of total GAGs. Surprisingly, no heparinoid was detected, but may be present in the fish at concentrations lower than the limit of the method used. HPLC of the disaccharides formed after chondroitin AC or ABC lyase degradation revealed that the zebrafish CS is composed by ΔUA-1→3-GalNAc($4SO_4$) (59.4%), ΔUA-1→3-GalNAc($6SO_4$) (23.1%), and ΔUA-1→3-GalNAc (17.5%) disaccharide units. No disulfated disaccharides were detected. Immunolocalization on sections from zebrafish retina using monoclonal antibodies against CS4- or 6-sulfate showed that in the retina these GAGs are restricted to the outer and inner plexiform layers. This is the first report showing the presence of KS in zebrafish eye, and the structural characterization of CS and its localization in the zebrafish retina. Detailed information about the structure and tissue localization of GAGs is important to understand the functions of these polymers in this model organism.

Keywords Chondroitin sulfate · Keratan sulfate · *Danio rerio* · Retina · Cornea · Disaccharide composition

The contributions of Aline R.C. Souza and Eliene O. Kozlowski should be considered equal.

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Abbreviations

α-ΔUA ($2SO_4$)	α-$\Delta^{4,5}$ unsaturated hexuronic acid 2-sulfate
α-ΔUA GalNAc ($4SO_4$)	α-$\Delta^{4,5}$ unsaturated hexuronic acid GalNAc($6SO_4$) and GalNAc(2,4-diSO_4), derivatives of *N*-acetyl-D-galactosamine, bearing a sulfate ester at position 4, at position 6, and at both positions 2 and 4, respectively
HPLC	high-performance liquid chromatography
FPLC	fast protein liquid chromatography

Introduction

Glycosaminoglycans (GAGs) are complex linear heteropolysaccharides formed by alternating disaccharide units, containing hexosamine (D-galactosamine or D-glucosamine) linked to hexuronic acid (D-glucuronic acid or L-iduronic acid) or galactose, which can be sulfated in various positions. With the probable exception of hyaluronic acid (HA), all other GAGs, including the galactosaminoglycans chondroitin sulfate (CS)/dermatan sulfate (DS), and the glucosaminoglycans heparan sulfate (HS)/heparin (Hep) and keratan sulfate (KS), occur in the tissues covalently linked to a polypeptide chain, forming the proteoglycans (PG). PGs are ubiquitously expressed in the tissues of vertebrates, occurring intracellularly, at the surface of virtually all types of cells or in the extracellular matrix [1–3].

KS was first identified in 1939 in cornea extracts [4], and characterized in 1950 as a linear polymer of lactosamine (3Galβ1-4GlcNAcβ1), sulfated at the carbon 6 of both hexoses [5]. KS is designated type I and type II based on differences between KS from cornea and that of cartilage. Corneal KS is N-linked to asparagine (Asn) residues in the core protein, whereas cartilage KS is O-linked to serine (Ser) or threonine (Thr) residues. Currently, the term KSI includes all Asn-linked KS molecules, and KSII is used to refer to all KS linked through GalNAc-O-Ser/Thr. There is also a third type of KS linkage (Mannose-*O*-Ser), which has been named KSIII [Reviewed in 6].

In zebrafish, PG and their GAGs chains, as well as several enzymes involved in the synthesis and modification of these polymers have been implicated in different molecular and cellular events during embryonic development and adult stage. UDP-glucose dehydrogenase, which is involved in the synthesis of HS, CS and HA, is required for ear development [7] and cardiac valve formation [8]. Enzymes of the biosynthesis (Ext-2 and Extl-3) and modification (HS6ST) of HS have been shown to participate in limb development [9] and in axon sorting in the optic tract [10], and in vascular [11] and muscle [12] development, respectively. In addition, HSPGs (glypican and syndecan) are essential for supporting gastrulating movements [13, 14] and for angiogenic sprouting [15]. Moreover, CS has been shown to participate in the formation of motor nerves [16] and axon guidance during embryonic development [17]. In the adult central nervous system of zebrafish, CS acts as an inhibitor of the neuronal growth, forming a repellent boundary, which prevents optic fibers from growing into inappropriate locations [18]. Although, these reports clearly indicate the presence of GAGs in zebrafish, very little is known about the disaccharide composition of these polymers in this organism.

In the present study, we performed a detailed structural analysis of the major GAGs isolated from the zebrafish *Danio rerio*. In addition, monoclonal antibodies were used to detect and localize specific structural motifs of the GAGs in the fish retina, in an attempt to correlate the structure and biological function of this polymer.

Results and discussion

Low-molecular-weight CS and keratan sulfate are the main GAGs isolated from Danio rerio The total sulfated GAGs obtained from the whole zebrafish after protease digestion, and standard CS, DS, HS and KS were analyzed by agarose gel electrophoresis, before or after incubation with chondroitin AC or ABC lyases, keratanase or treatment with nitrous acid. Two well-distinguished metachromatic bands with different motilities were observed in the gel after electophoresis of the total GAGs from zebrafish (Fig. 1a). The lower mobility band migrates between standard DS and HS, and corresponds to ~20% of the total polysaccharides (Fig. 1a and c). This material resisted incubation with chondroitin AC and ABC lyases, and deaminative cleavage with nitrous acid, but was completely degraded by keratanase treatment, indicating that it is a KS GAG (Fig. 1a). The higher mobility band migrated as standard CS and represents about 80% of the total polysaccharides (Fig. 1a and c). This material resisted deaminative cleavage with nitrous acid, but was completely degraded by chondroitin AC and ABC lyases, indicating that this polymer is a CS GAG. Incubation of standard GAGs with Chase AC/ABC, keratanase and nitrous acid treatment abolished the metachromatic bands of the GAGs in the agarose gel, indicating that the enzymes were completely active (Fig. 1b). No other metachromatic band was detected in the agarose gel, which is a very sensitive method for the detection as low as 100 ng of sulfated GAGs. This implies that CS and KS are the main sulfated GAGs present in *Danio rerio*. Although several enzymes involved in the synthesis of HS have been described in zebrafish embryos [7, 9], there have been no reported investigations of HS in the adult fish. Our results, however, do not exclude the occurrence of smaller amounts of HS or other non-sulfated GAGs in adult zebrafish. In fact, in vertebrates, HS and HA participate in important biological and cellular events that occur not only during development but also in the adult [see 1–3 for review].

The total sulfated GAGs extracted from adult zebrafish were partially purified on a DEAE-cellulose column eluted stepwise with 2 M NaCl (not shown). The DEAE-purified sulfated glycans (CS and KS) were re-chromatographed on the same column and eluted with a linear NaCl gradient (0–2.0 M). Two symmetric metachromatic peaks, denominated P1 and P2, eluted from the column with ~0.5 and 1.0 M NaCl, respectively (Fig. 1c). Analysis by agarose gel electrophore-

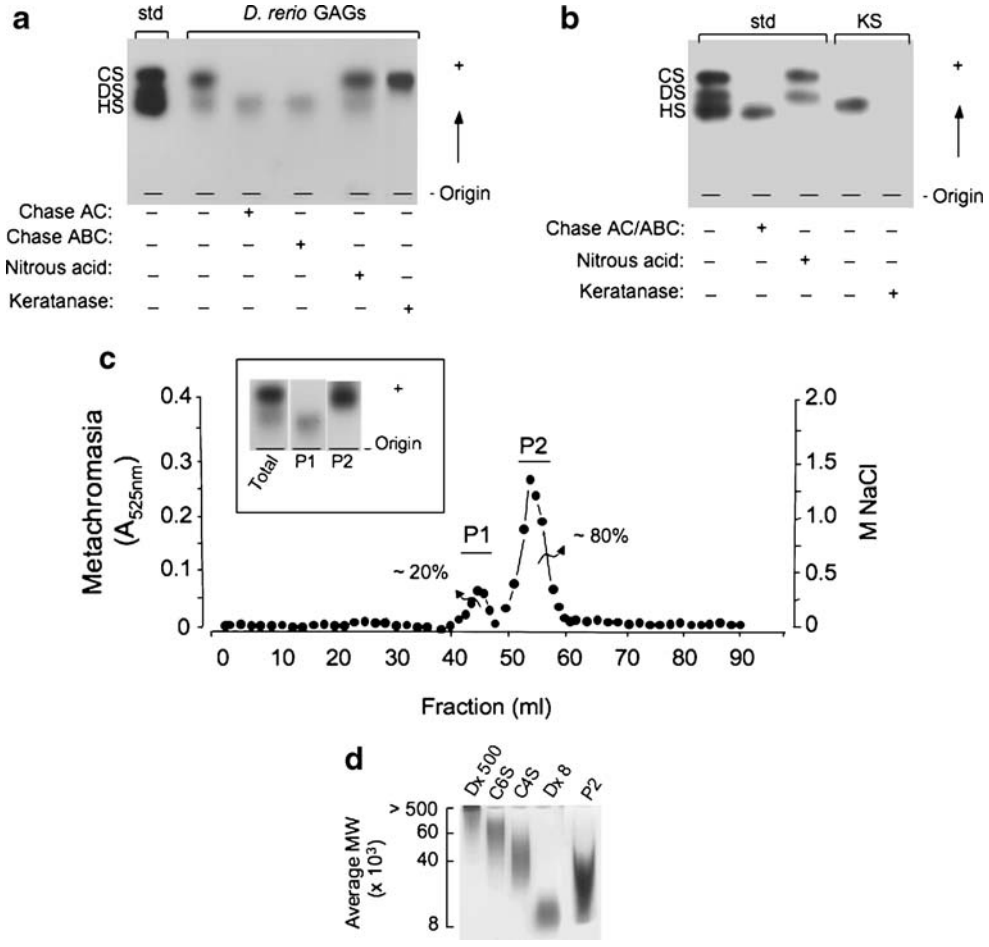

Fig. 1 Biochemical analysis and fractionation of the sulfated glycans isolated from *Dario rerio*. **a** The total glycans from zebrafish (~1.5 μg as uronic acid), before (−) or after (+) incubation with chondroitin AC (Chase AC) or ABC (Chase ABC) lyase, keratanase or deaminative cleavage with nitrous acid, as well as a mixture of standard GAGs, containing CS, DS and HS (1.5 μg as uronic acid of each) were analyzed by agarose gel electrophoresis as described in the Materials and methods section. **b** Agarose gel electrophoresis of standard GAGs before (−) or after (+) enzymatic incubation or deaminative cleavage with nitrous acid. **c** About 60 mg of the DEAE-cellulose-purified glycans (see Material and methods section) were fractionated on a DEAE-cellulose column, eluted with a linear gradient of 0–2 M NaCl. Fractions were assayed by metachromasia (*lambda symbol*) and NaCl concentration (*dotted line*). The fractions eluted under the peaks denominated P1 and P2 were pooled, dialyzed against distilled water and analyzed by agarose gel electrophoresis (*insert*). The percentage of P1 and P2 was estimated by integrating the areas under the peaks. **d** P2 and the molecular weight markers dextran 500, chondroitin 4-sulfate; chondroitin 6-sulfate and dextran 8 (~10 μg as dry weight of each) were applied to a 1-mm thick 6% polyacrylamide slab gel, as described in the Materials and methods section

sis revealed that P1 and P2 correspond to KS and CS, respectively (Fig. 1c, insert). The identity of the purified glycans was confirmed by incubation with chondroitin AC lyase, keratanase and nitrous acid treatment (not shown).

In order to estimate the molecular weight of the zebrafish CS, the purified glycan obtained after the DEAE-cellulose column (P2) was analyzed by polyacrylamide gel electrophoresis, and its migration compared with those of known molecular weight standard glycans. As shown in Fig. 1d, the CS from the fish migrated as a very polydisperse metachromatic band, slightly behind dextran 8,000 (average MW 8,000 Da), suggesting an average molecular weight of ~10,000 Da. This result indicates that the CS chains in *Danio rerio* are shorter than those of chondroitin 4-sulfate from whale cartilage (average MW 40,000) and chondroitin 6-sulfate from shark cartilage (average MW 60,000) and close to that of the hybrid CS/DS from hagfish notochord [19]. However, different from the hybrid glycan from hagfish, the zebrafish galactosaminoglycan has only D-glucuronic acid-containing disaccharides, since it is completely degraded by chondroitin AC lyase (Figs. 1a and 2a). At present, we cannot speculate whether these differences are due to phylogenetic of physiological reasons.

The zebrafish CS is composed by 0-, 4- or 6-mono-sulfated units The purified CS from zebrafish obtained after ion-exchange chromatography was exhaustively degraded with chondroitin AC and ABC lyase and the disaccharides

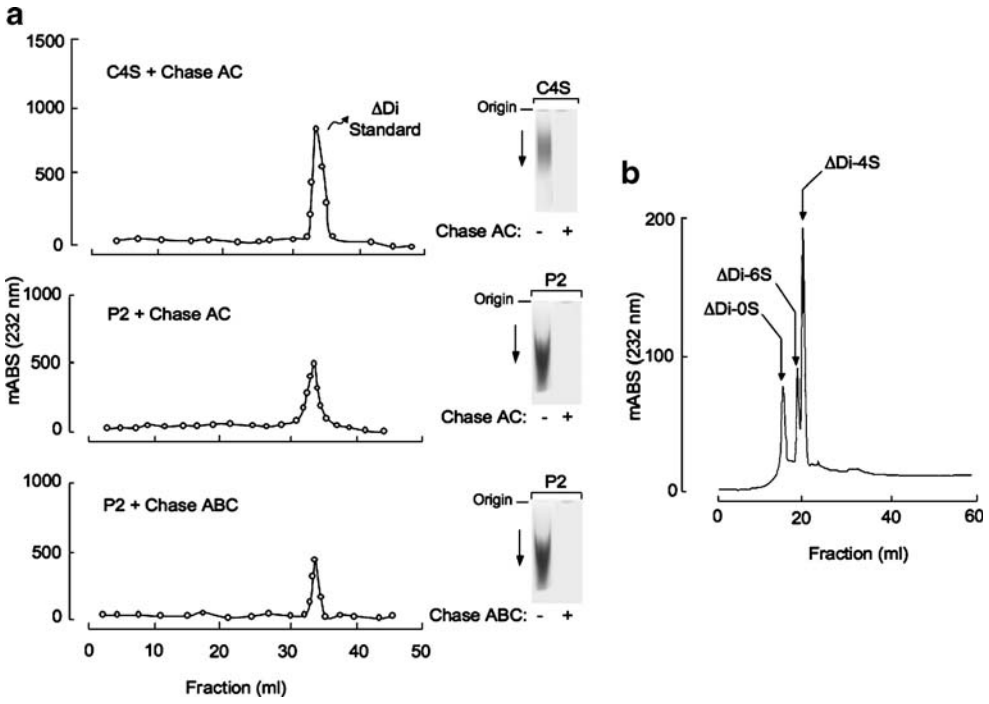

Fig. 2 Analysis of the products formed by degradation of the purified zebrafish CS with chondroitin AC/ABC lyases. **a** Standard CS or the purified zebrafish CS was incubated with chondroitin AC or ABC lyases, as described in the Materials and methods section. The incubation products and a standard ΔDi4-S disaccharide were applied on a Superdex peptide-column. Fractions of 0.25 ml were collected and monitored for UV absorbance at 232 nm. Fractions corresponding to disaccharides (>95 % of the degraded material) were pooled and lyophilized. Intact (−) or enzyme-degraded (+) standard CS or the purified zebrafish CS were analyzed by polyacrylamide gel electrophoresis (insert, Fig. 2a). **b** The disaccharides obtained after chondroitin AC lyase treatment of the zebrafish CS, eluted in fractions (31–35) from the peptide column, and the standard disaccharides ΔDi0-S, ΔDi4-S, ΔDi6-S, and ΔDi2,4-S were applied on a SAX-HPLC analytical column, and eluted with a linear gradient of 0 –1.0 M NaCl, as described in the Materials and methods section. The eluant was collected in 0.5 ml fractions and monitored for UV absorbance at 232 nm for comparison with the standard disaccharides. The percentage of disaccharides was estimated by integrating the areas under the peaks. The *arrows* indicate the position of elution of the standard disaccharides

formed were separated by gel filtration chromatography on a Peptide column. As shown in Fig. 2a, the glycan was completely degraded by the enzymes yielding an unique symmetric peak eluting at the same position as standard unsaturated mono-sulfated disaccharides. Moreover, no low molecular weight oligosaccharides were detected by polyacrylamide gel electrophoresis analysis of the chondroitin AC/ABC lyase products (Fig. 2a). The disaccharide mixture was then analyzed on a strong anion-exchange HPLC, together with the standard disaccharides: ΔUA-1→3-GalNAc, ΔUA-1→3-GalNAc($6SO_4$), ΔUA-1→3-GalNAc($4SO_4$) and ΔUA($2SO_4$)-1→3-GalNAc($4SO_4$). The analysis revealed that the action of the chondroitin AC lyase on the zebrafish CS yielded the mono-sulfated disaccharides ΔUA-1→3-GalNAc($4SO_4$) (59.4%) and ΔUA-1→3-GalNAc($6SO_4$) (23.1%), and the non-sulfated disaccharide ΔUA-1→3-GalNAc (17.5%) (Fig. 2b, and Table 1). No disulfated disaccharides were detected. About 96.6% of the disaccharides (as μg of uronic acid) applied to the ion-exchange HPLC column was recovered as disaccharides peaks (Table 1). The proportions of the different disaccharides estimated by uronic acid, are very close to that obtained by integrating the area of the peaks. This indicates that no other undetected disaccharide was present in the sample.

Table 1 Total uronic acid recovered as ΔDisaccharides after ion-exchange HPLC column

Total Disaccharide applied to the HPLC column (μg of uronic acid)	ΔDi recovered (μg of uronic acid)		
65	ΔUA-1→4-β-D-GalNAc	ΔUA-1→4-β-D-GalNAc ($6SO_4$)	ΔUA-1→4-β-D-GalNAc ($4SO_4$)
	9.9 (15.2)[a]	16.2 (24.9)[a]	36.7 (54.8)[a]

[a] Value represents the percentage of the disaccharide estimated by integrating the peaks in Fig. 2b.

In order to obtain more information about the structure of the chondroitin sulfate chains, a short-term partial chondroitin AC-lyase treatment were performed to obtain oligosaccharide mixtures. Five symmetric uronic acid-containing peaks were obtained (Fig. 3a). When analyzed by polyacrylamide gel-electrophoresis, the peaks showed homogeneous metachromatic bands, with electrophoretic motilities compatible with the presence of oligosaccharides corresponding to deca (F10), octa (F8), hexa (F6) and tetra (F4) units (Fig. 3b). Fraction F2 was not loaded onto the gel because it did not show metachromatic property, which is compatible with the presence of disaccharides. Tetra and hexasaccharides were then isolated by gel filtration chromatography, submitted to long-term total chase AC treatment and the disaccharides formed analyzed by HPLC (Fig. 3c). Complete degradation of the tetrasaccharides produced the disaccharides: ΔUA-1→3-GalNAc($4SO_4$); ΔUA-1→3-GalNAc($6SO_4$) and ΔUA-1→3-GalNAc in the proportion of 2 : 1 : 0.5. The same disaccharides were obtained after complete degradation of the hexasaccharides, but in a different proportion (1 : 1 : 0.3).

Analysis of the oligosaccharide data is complex and may have different interpretations. One possibility that can be supported by the data is the following: considering that the average molecular weight of the chondroitin sulfate chains is about 10.000 Da (estimated by polyacrylamide gel on Fig. 1d), the preponderant chain would have ten repeating disaccharide units (Fig. 4) and would contain homogeneous sequences composed by ΔUA-1→3-GalNAc($4SO_4$) units, alternating with a shorter homogeneous sequence formed by ΔUA-1→3-GalNAc($6SO_4$). The non-sulfated ΔUA-1→3-GalNAc unit would mostly be present in small quantity in the GalNAc($6SO_4$)-containing sequences. This arrangement would explain the observed disaccharide proportions obtained by complete degradation of the tetra and hexasaccharides (Fig. 4).

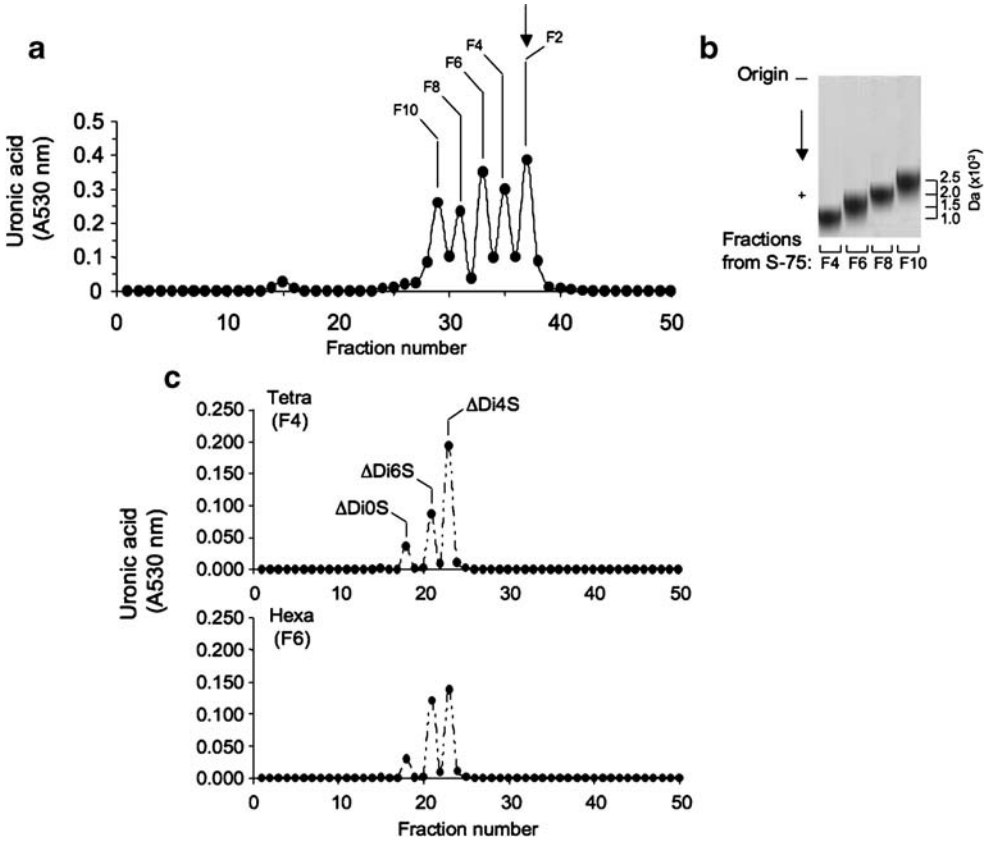

Fig. 3 Characterization of tetra- and hexasaccharides prepared by short-term partial degradation of the zebrafish chondroitin sulfate. **a** the purified zebrafish CS was incubated with chondroitin AC lyase for 20 min at 37°C, as described in the Materials and methods section. The incubation products and a standard ΔDi4-S disaccharide were applied on a Superdex 75-column. Fractions of 0.25 ml were collected and monitored for uronic acid. **b** Fractions corresponding to oligosaccharides of different sizes, deca (F10), octa (F8), hexa (F6), tetra (F4) were analyzed by 12% polyacrylamide gel electrophoresis, as described in the Materials and methods section. **c** The disaccharides obtained after long-term chondroitin AC lyase degradation of the tetra- and hexasaccharides from the Superdex 75-column, and the standard disaccharides ΔDi0-S, ΔDi4-S, ΔDi6-S (not shown), were applied on a SAX-HPLC analytical column, and eluted with a linear gradient of 0–1.0 M NaCl, as described in the Materials and methods section. The eluant was collected in 0.5 ml fractions and monitored for the content of uronic acid for comparison with the standard disaccharides. The proportion of disaccharides was estimated by integrating the *areas under the peaks*. *Arrow* in **a** represents the position of elution of 4-sulfated disaccharide

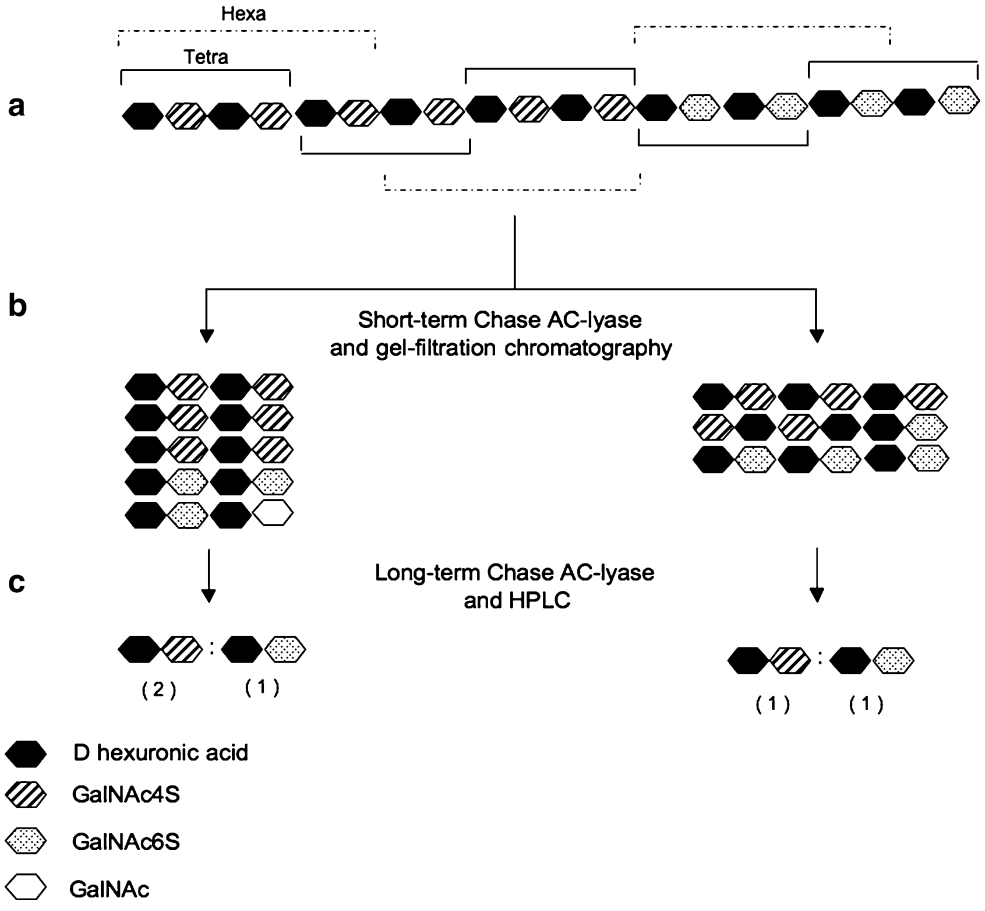

Fig. 4 Proposed structure of the average zebrafish chondroitin sulfate chain based on oligosaccharide analysis. **a** the preponderant chain would have ten repeating disaccharide units arranged in homogeneous sequences composed by ΔUA-1→3-GalNAc($4SO_4$) units, alternating with a shorter homogeneous sequence formed by ΔUA-1→3-GalNAc($6SO_4$). The non-sulfated ΔUA-1→3-GalNAc unit would mostly be present in small quantity in the GalNAc($6SO_4$)-containing sequences. **b** Tetra- and hexasaccharides formed by short-term partial Chase AC lyase digestion. **c** Disaccharides produced by long-term total Chase AC lyase digestion. *Number in parenthesis* represents the proportion of the disaccharide units

To our knowledge, this is the first report on the analysis of the GAGs isolated from adult *Danio rerio*. Although previous studies reported the presence of CS in embryos and adult individuals of this species, using specific antibodies [16–18], no information about the disaccharide composition was provided.

KS is the major GAG in the eye In vertebrates, KS is an abundant component of the cornea, occurring in a concentration tenfold higher than that found in cartilage [6]. To investigate the GAGs in the zebrafish eye, the eyes of several individuals we carefully isolated after anesthesia, and subjected to proteolytic digestion. The extracted GAGs represented about 14.4% of the dry weight of the cornea, and agarose gel electrophoresis before and after incubation with keratanase and Chase AC (not shown) indicated the occurrence of KS and CS (Fig. 5a), in a proportion of 80.5 and 19.5%, respectively (Fig. 5b). It remains to be determined if the KS from the zebrafish cornea is the prototype for KS type I found in vertebrate cornea [6].

Chondroitin 4 - and 6-sulfate are present in the retina of zebrafish CS has been previously reported in the visual system of adult zebrafish, occurring in retina, optic nerve, chiasm, optic tract, tectum and other targets of optic axons [18]. To confirm the presence of CS containing ΔUA-1→3-GalNAc($4SO_4$) and ΔUA-1→3-GalNAc ($6SO_4$) units in the retina, we used monoclonal antibodies that recognize specific epitopes generated by the action of chondroitin ABC lyase on sections from adult zebrafish. Chondroitin 4- and 6-sulfate epitopes were strongly detected in the outer and inner plexiform layers (Fig. 5c). No positive reaction was observed in control experiments where the primary antibodies were omitted (not shown). The inner and outer plexiform layers are nerve-rich layers where vertical-oriented axonal projections connect different neuronal cells to integrate the signals to be transmitted to the brain by the optic nerve. Recent studies revealed that phosphacan, a nervous system-specific CSPG is localized around parallel neuronal fibers and not at the synapses, modulating axonal extension in rat cerebellum [20]. In zebrafish, CS contributes to axon guidance of the optic projection [18].

Therefore, in the light of the results presented in the present study, it is possible to suggest that in *Danio rerio*, CS sequences enriched in 4- or 6-mono sulfated disaccharides participate in axon guidance in the retina. It has been suggested that the effect of CS on neurons involves

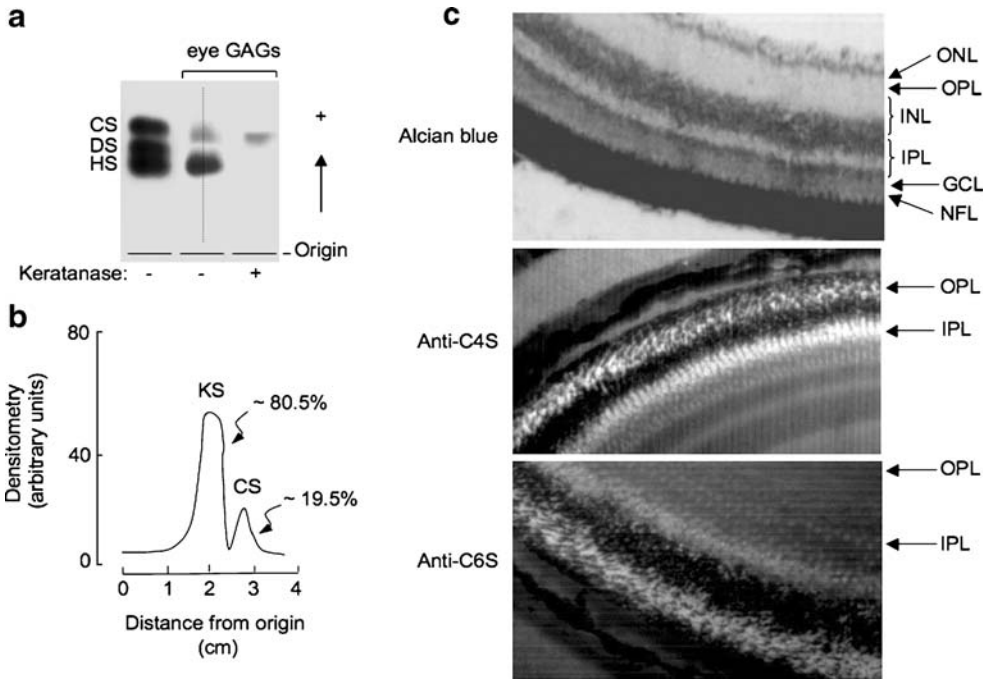

Fig. 5 Characterization of the GAGS from the eye and immunolocalization of chondroitin 4- and 6-sulfate epitopes in the zebrafish retina. **a** Agarose gel electrophoresis of the GAGs extracted from the zebrafish eye before (−) or after (+) keratanase digestion. **b** Densitometric analysis of the metachromatic bands on the agarose gel shown in Fig. 3a. **c** Sections from adult retina were incubated with chondroitin ABC lyase to unmask specific chondroitin 4- and 6-sulfate antigen epitopes and then incubated with the primary anti-chondroitin 4- or 6-S antibodies, as described in the Materials and methods section. The sections were analyzed by a fluorescent microscope, and the *brilliant white spots* represent positive reaction (*middle and bottom panels*). For the identification of the different layers of the zebrafish retina, the sections were stained with alcian blue prior incubation with chase ABC, and analyzed by light microscope (*upper panel*). *ONL* Outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer

the binding and regulation of different heparin-binding neurotrophic factors [21, 22]. However, it remains to be determined if the zebrafish CS binds to neurotrophic factors and modulate their activities.

In conclusion, CS and KS were isolated from the tissues of the zebrafish *Danio rerio*. CS has a low-molecular-weight, and consisted of 4-sulfated, 6-sulfated and non-sulfated disaccharide units. In the retina, 4- and 6-sulfated units were restricted to the outer and inner plexiform layers. KS was shown to be the major GAG in the eye, probably occurring in the cornea. Detailed information about the structure and the tissue localization of GAGs can establish the starting point to understand the functions of these polymers in this model organism.

Materials and methods

Materials HS from human aorta was extracted and purified as described previously [23]. Chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, DS from bovine intestinal mucosa, twice-crystallized Papain (15 units/mg protein) and the standard disaccharides α-ΔUA-1→3-GalNAc (ΔDi0S), α-ΔUA-1→3-GalNAc ($6SO_4$) (ΔDi6S), α-ΔUA-1→3-GalNAc($4SO_4$) (ΔDi4S), and α-ΔUA($2SO_4$)-1→3-GalNAc($4SO_4$) (ΔDi2,4S), were purchased from Sigma (St. Louis, MO, USA); chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens*, chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, keratanse (EC 3.2.1.103) from *Pseudomonas* and keratan sulfate from bovine cornea, monoclonal anti-chondroitin Ddi-4S (2-B-6) and monoclonal anti-chondroitin Ddi-6S (3-B-3) were from Seikagaku America Inc. (Rockville, MD, USA); agarose (standard low M_r) was from Bio-Rad (Richmond, CA, USA); Toluidine blue was from Fisher Scientific (NJ, USA) and 1,9-dimethylmethylene blue from Serva Feinbiochimica (Heidelberg, Germany); cetyltrimethylammonium bromide from Merk (Darmstadt, Germany) and DEAE-cellulose (DE-52) from Whatman International (Maidstone, UK);

Animals Adult (body length > 2 cm, age > 4 months) zebrafish, *Danio rerio*, were bought at a local pet shop and maintained in an aerated aquarium at a 12/12 h light/dark cycle and a temperature of 27°C until use. Fish were fed a

dried fish food, consisting of Brine shrimp. Prior to the experimental procedures, the animals were anesthetized by lowering the temperature to 4°C and then sacrificed by placing them in cold 99.5% acetone.

Purification of the sulfated glycans The sulfated glycans were extracted from dried zebrafishes (20 g) or from isolated eyes (0.24 g) by papain digestion, as described previously [24]. The papain-extracted glycans from the body (200 mg) were then applied to a DEAE-cellulose column (10× 1.5 cm), equilibrated with 50 mM sodium acetate (pH 5.0). The column was washed with 50 ml of 50 mM sodium acetate (pH 5.0) and eluted stepwise with 50 ml of 2.0 M NaCl in the same buffer. The sulfated glycans eluted with 2.0 M NaCl were dialyzed against distilled water and lyophilized. The lyophilized powder was then suspended in 2 ml phosphate-buffered saline containing approximately 0.5 mg deoxyribonuclease I (Sigma St. Louis, USA) and incubated for 12 h at 37°C. The incubation mixture was lyophilized and dissolved in distilled water.

The DEAE-cellulose-purified glycans from zebrafish (~60 mg) were reapplied to a DEAE-cellulose column (10×1.5 cm), equilibrated with 50 mM sodium acetate (pH 5.0). The column was developed by a linear gradient of 0–2.0 M NaCl in the same buffer. The flow rate of the column was 0.5 ml/min, and fractions of 1 ml were collected and assayed by metachromasia using 1,9-dimethylmethylen blue [25]. The fractions containing the sulfated glycans were pooled, dialyzed against distilled water and lyophilized.

Electrophoretic analysis

Agarose gel The crude or purified glycans from the whole zebrafish or from the eyes, as well as standard GAGs, before or after incubation with specific GAG lyases (Chase AC, Chase ABC or Keratanse), or deaminative cleavage with nitrous acid were analyzed by agarose gel electrophoresis, as described previously [26]. Briefly, about 1.5 μg (as uronic acid) of the glycans, and a mixture of standard GAGs, containing CS, DS, HS and KS (1.5 μg as uronic acid of each) were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane/acetate (pH 9.0), and run for 1 h at 110 mV. After electrophoresis the glycans were fixed with aqueous 0.1% cetylmethylammonium bromide solution and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, *v*/*v*). The proportion of the GAGs in the eye was estimated by densitometry of the metachromatic bands after agarose gel electrophoresis of the total glycans extracted from the eye.

Polyacrylamide gel The molecular mass of the purified sulfated glycan from zebrafish was estimated by polyacrylamide gel electrophoresis. Samples (~10 μg as dry weight) were applied to a 1-mm thick 6% polyacrylamide slab gel, and after electrophoresis at 100 V for ~1 h in 0.06 M sodium barbital (pH 8.6), the gel was stained with 0.1% toluidine blue in 1% acetic acid. After staining, the gel was washed overnight in 1% acetic acid. The molecular mass markers used were: dextran 500 (average MW 500,000), chondroitin 4-sulfate from whale cartilage (average MW 40,000); chondroitin 6-sulfate from shark cartilage (average MW 60,000) and dextran 8 (average MW 8,000). Analysis of the oligosaccharides obtained by short-term partial degradation with Chase AC lyase was carried out in a 1-mm thick 12% polyacrylamide slab gel.

Enzymatic treatment

Chondroitin lyases The zebrafish glycans (~100 μg) were incubated with 0.01 U of chondroitin AC or ABC lyase (Seikagaku, Japan) in 0.1 ml 50 mM Tris–HCl buffer (pH 8.0), containing 5 mM EDTA and 15 mM sodium acetate. After incubation at 37°C for 12 h, another 0.01 U of enzyme was added to the mixture, and the reaction continued for an additional 12-h period. At the end of the incubation period the mixtures were analyzed by agarose gel electrophoresis, or by HPLC after purification of the disaccharides. Short-term partial degradation was achieved by incubating the chondroitin sulfate chains with 0.01 U of chondroitin AC lyase for 20 min at 37°C, as described above. The oligosaccharides formed were fractionated on a Superdex 75 column (Amersham Pharmacia Biotech) linked to a HPLC system from Shimadzu (Tokyo, Japan). The column was eluted with distilled water:acetonitrile: trifluoroacetic acid (80:20:0.1, *v*/*v*) at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and monitored for uronic acid by the carbazole reaction [27].

Keratanase The zebrafish glycans (~100 μg), or standard KS were incubated with 0.01 U of keratanse in 0.1 ml 10 mM Tris–HCl buffer (pH 7.4). After incubation at 37°C for 30 min, another 0.01 U of enzyme was added to the mixture, and the reaction continued for an additional 30-min period. At the end of the incubation period the mixtures were analyzed by agarose gel electrophoresis.

Disaccharide analysis In order to determine the disaccharide composition, the purified sulfated glycan from zebrafish or the tetra- and hexasaccharides were incubated with chondroitin AC lyase, as described earlier. Disaccharides were recovered by a Superdex peptide-column (Amersham Pharmacia Biotech) linked to a HPLC system from Shimadzu (Tokyo, Japan). The column was eluted with distilled water:acetonitrile:trifluoroacetic acid (80:20:0.1, *v*/*v*)

at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and monitored for UV absorbance at 232 nm. Fractions corresponding to disaccharides (>95% of the degraded material) were pooled, freeze–dried and stored at −20°C. The extent of degradation was analyzed by polyacrylamide gel electrophoresis as described earlier. The disaccharide preparation and standard disaccharides (Sigma, St. Louis, USA) were subjected to a SAX-HPLC analytical column (250×4.6 mm, Sigma-Aldrich), as follows. After equilibration in the mobile phase (distilled water adjusted to pH 3.5 with HCl) at 0.5 ml/min, samples were injected and disaccharides eluted with a linear gradient of NaCl from 0 to 1.0 M over 45 min in the same mobile phase. The eluant was collected in 0.5 ml fractions and monitored for UV absorbance at 232 nm for comparison with lyase-derived disaccharide standards.

Hexuronic acid The hexuronic acid content of the glycans was estimated by the carbazole reaction [27].

Deaminative cleavage with nitrous acid Deaminative cleavage with nitrous acid of the sulfated glycans was performed at pH 1.5, as described previously [28].

Immunohistochemistry Adult zebrafishes were anesthetized by lowering the temperature to 4°C and fixed in a formalin solution for 4 h at room temperature. The samples were then immersed in 10% EDTA, 10% formaldehyde (pH 7.4) for 2 weeks at room temperature. After the decalcification period, the samples were washed with distilled water, dehydrated in graded ethanol, cleared in xylol, and embedded in Para-plast (m.p. 55.6°C). Approximately 7-μm sections from the whole fish were cut horizontally on a Spencer microtome and permeabilized with 0.5% Triton X-100 in PBS for three times (5 min each) at room temperature. For immunostaining of the eye, using chain-specific chondroitin antibodies against chondroitin 4- or 6-sulfate (Seikagaku, Tokyo, Japan), the reaction with primary antibodies was preceded by digestion with chondroitinase ABC (Seikagaku) to unmask the specific antigen epitope. Sections were incubated with 0.5 U of chondroitinase ABC in 1 ml 0.25 M Tris–HCl buffer (pH 8.0) for 1 h at 37°C in a moist chamber. The slides were carefully washed in 0.1 M phosphate buffered saline (PBS), followed by 50 mM NH_4Cl and PBS, and incubated with PBS, 1% bovine serum albumin in PBS (pH 7.4) for 1 h. This procedure avoids nonspecific binding of antibody. The sections were then incubated with the primary antibodies (dilution 1:100) overnight at 4°C in a moist chamber, washed with PBS and incubated with a FITC-conjugated secondary antibody (mouse IgG) for 45 min at 37°C in a moist chamber. The sections were then mounted in glycerol and examined under an optical microscope Axiovert 100 (Carl Zeiss, Germany). High-resolution images were obtained with a CCD camera (Hamamatsu Photonics, Japan) coupled to the image acquisition system Argus 20 (Hamamatsu Photonics, Japan). Controls were obtained by omitting the primary antibodies.

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