The core molecule from type H proteoglycan

Release of mannose-containing oligosaccharides by digestion with *N*-oligosaccharide glycopeptidase

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Chick-embryo cartilage contains a unique set of proteoglycans. Type H proteoglycan (PG-H) is the most abundant, constituting over 90% of the total cartilage hexuronate. We previously showed that treatment of PG-H with chondroitinase ACII and keratanase yields a protein-enriched core molecule [PG(–CS,KS)] with enzymically modified linkage oligosaccharides of the chondroitin sulphate and keratan sulphate chains. We report here that further treatment of PG(–CS,KS) with pepsin and *N*-oligosaccharide glycopeptidase (almond glycopeptidase) released four distinct types of mannose-containing oligosaccharide. Two of them were shown to be:

Of the mannose-containing glycopeptides formed by pepsin digestion, about 40% (as mannose) were resistant to *N*-oligosaccharide glycopeptidase. Since the resistant fraction was enriched in keratan sulphate remnants, it is suggested that the mannose-containing oligosaccharides in this fraction represent those located in a keratan sulphate-enriched region of PG-H.

The proteoglycan synthesized by chick-embryo epiphyseal cartilages or by limb-bud chondrocyte cultures has in recent years been the subject of intensive investigation (for reviews see Muir,

Abbreviations used; PG-H, type H proteoglycan (the predominant proteoglycan of chick-embryo cartilages); PG(–CS,KS), protein-enriched core molecule obtained from PG-H by selective removal of the chondroitin sulphate and keratan sulphate chains with chondroitinase ACII and keratanase respectively; SDS, sodium dodecyl sulphate.

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1980; Hascall & Hascall, 1981), and it is now certain that there are at least three distinct molecular species (designated type H, Lb and Lt proteoglycans) (Noro *et al.*, 1983; Shinomura *et al.*, 1983, 1984). Type H proteoglycan (PG-H) is the most abundant, constituting about 94% of the total proteoglycan hexuronate or 65% of the total proteoglycan serine of the epiphyseal cartilage of 12-day chick embryo. It is believed that this type of proteoglycan plays a role in processes of matrix organization, which involve interaction with other macromolecules such as hyaluronic acid and link protein (Hascall *et al.*, 1976; De Luca *et al.*, 1977).

In studies on the proteoglycan from chick limb-bud chondrocyte cultures, De Luca *et al.* (1980) showed that oligosaccharide-peptides containing mannose, *N*-acetylglucosamine, galactose and sialic acid were obtained after alkaline-borohydride treatment of the proteoglycan fraction. Although the results suggest that *N*-linked oligosaccharides occur in the proteoglycan molecule, their precise structures remain to be determined. More recently, Geetha-Habib *et al.* (1984) showed that, when chick-embryo chondrocytes were pulse-labelled with either [³H]mannose or [³H]glucosamine, *N*-linked oligosaccharide addition was found on the earliest synthesized core-protein precursor. The oligosaccharide was susceptible to cleavage by endo- β -*N*-acetylglucosaminidase H, and upon 'chase' about 56 and 31% of the glucosamine-labelled and mannose-labelled oligosaccharides respectively were processed to resistant forms. To understand thoroughly the structure, processing and function of cartilage proteoglycans, structural studies on the *N*-linked oligosaccharide chains are clearly needed.

We previously described enzymic procedures suitable for selective removal of the chondroitin sulphate and keratan sulphate, without altering the core-protein region of the macromolecule (Oike *et al.*, 1980). The resultant core fraction, PG(-CS,KS), was shown to contain a single non-variable polypeptide chain (Oike *et al.*, 1982) and a number of carbohydrate chains including residual linkage oligosaccharides of the glycosaminoglycan chains (Oike *et al.*, 1980). In the present work, we examined the possibility that the core molecule may contain *N*-glycosidically linked oligosaccharides. We utilized *N*-oligosaccharide glycopeptidase (EC 3.5.1.52; formerly called almond glycopeptidase), the enzyme capable of hydrolysing β -aspartyl-*N*-acetylglucosylamine linkages in glycoproteins or glycopeptides (Takahashi & Nishibe, 1978), to release *N*-linked oligosaccharides from the peptide portion. The released oligosaccharides were analysed by a simple rapid procedure with thin-layer plates that is applicable to ultramicro quantities. The results indicated that at least four distinct types of mannose-containing oligosaccharides chains occur in PG(-CS,KS).

Experimental

Materials

PG-H and its protein-enriched core fraction, PG(-CS,KS), were prepared from epiphyseal cartilages from 12-day chick embryos as previously described (Oike *et al.*, 1980). The isomaltoligosaccharides of 4-14 glucose units were prepared from the acid hydrolysate of dextran by the

method of Nishigaki *et al.* (1978). The high-mannose-type oligosaccharides Man₅₋₆GlcNAc₂ (Table 1, compounds A and B) and the hybrid-type oligosaccharides Gal₀₋₁Man₄₋₅GlcNAc₄₋₅ (Table 1, compounds F-J) are those described by Tai *et al.* (1975, 1977) and Yamashita *et al.* (1978) and were isolated, and identified, from ovalbumin by the method of Ishihara *et al.* (1981). The high-mannose-type oligosaccharides Man₇₋₉GlcNAc₂ (Table 1, compounds C-E) are those described by Tsuji *et al.* (1981) and were isolated, and identified, from pig thyroglobulin (kindly given by Dr. O. Tarutani, Gunma University, Gunma, Japan) by the same method as used for the oligosaccharides from ovalbumin (Ishihara *et al.*, 1981). Chondroitinase ACII from *Arthrobacter aureus* (Hiyama & Okada, 1975), keratanase from *Pseudomonas* (Nakazawa & Suzuki, 1975) and *N*-oligosaccharide glycopeptidase (commercial name glycopeptidase A) from almond nuts (Takahashi & Nishibe, 1978) are products of Seikagaku Kogyo Co. (Tokyo, Japan) and were kindly donated by Mr. M. Mizutani of the company. These commercial enzyme preparations are available from the above company or from Miles Laboratories (Elkhart, IN, U.S.A.). Pronase P was a product of Kaken Seiyaku Co. (Tokyo, Japan) and was kindly donated by Mr. K. Takamine of the company. D-[2-³H]Mannose (16 Ci/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.); tissue-culture materials were from Grand Island Biological Co. (Grand Island, NY, U.S.A.); bovine serum albumin (fraction V) and pepsin (3900 units/mg) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); crystalline papain was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); Bio-Gel P-4 (200-400 mesh) was from Japan Bio-Rad Laboratories (Tokyo, Japan); silica gel 60 plates (reference no. 5553) were from E. Merck (Darmstadt, West Germany); silicone OV-101 (G-SCOT) was from Gasukuro Kogyo Co. (Tokyo, Japan); chemicals for the preparation of electrophoresis gels were from Nakarai Chemicals (Kyoto, Japan).

Preparation of [³H]mannose-labelled PG(-GS,KS)

For each incubation, about 1.2 g of cartilages (tibiae and femurs from 12-day chick embryos) was placed in a 100 ml conical flask containing 6 ml of incubation medium. The medium was a modified Eagle's minimal essential medium containing 10% (v/v) foetal-calf serum, 0.005% ascorbic acid and 167 μ Ci of D-[2-³H]mannose. Incubation was carried out for 5 h in a humidified atmosphere containing 5% CO₂ at 37°C with gentle shaking. At the end of the incubation period, the tissues were transferred to 1 l ml of fresh medium with no radioactive precursor, incubated for 5 min and then re-

Table 1. Structures of oligosaccharides

Compound	Monosaccharide composition	Structure
A	Man ₅ GlcNAc ₂	Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
B	Man ₆ GlcNAc ₂	Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3
C	Man ₇ GlcNAc ₂	Man α 1 $\rightarrow$ 2{ Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
D	Man ₈ GlcNAc ₂	(Man α 1 $\rightarrow$ 2) ₂ { Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
E	Man ₉ GlcNAc ₂	Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
F	Man ₅ GlcNAc ₄	GlcNAc β 1 Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 GlcNAc β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
G	Man ₄ GlcNAc ₅	GlcNAc β 1 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 GlcNAc β 1 $\rightarrow$ 4Man α 1 $\rightarrow$ 3 GlcNAc β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
H	Man ₅ GlcNAc ₅	GlcNAc β 1 Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 GlcNAc β 1 $\rightarrow$ 4Man α 1 $\rightarrow$ 3 GlcNAc β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
I	Gal ₁ Man ₄ GlcNAc ₅	GlcNAc β 1 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4Man α 1 $\rightarrow$ 3 GlcNAc β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc
J	Gal ₁ Man ₅ GlcNAc ₅	GlcNAc β 1 Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ 3 Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6 Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4Man α 1 $\rightarrow$ 3 GlcNAc β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3 Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc

placed in the same volume of the washing medium for an additional incubation of 5 min.

From the ^3H -labelled cartilages, radioactive PG-H was prepared in accordance with the preparative sequence shown in our previous paper (Oike *et al.*, 1982). About $40\text{ }\mu\text{mol}$ (as glucuronic acid), 1.2 mg (as protein based on the Lowry assay) or $1.7 \times 10^6\text{ d.p.m.}$ (as ^3H) of PG-H was obtained from 1.2 g of the labelled tissues. The [^3H]PG-H preparation was then treated with chondroitinase ACII and keratanase, and the resultant protein-enriched core molecule, PG(-CS,KS), was isolated from the digest as previously described (Oike *et al.*, 1982). About $620\text{ }\mu\text{g}$ (as protein) or $6.2 \times 10^5\text{ d.p.m.}$ (as ^3H) of PG(-CS,KS) was obtained.

Analysis of the ^3H -labelled PG(-CS,KS) sample was carried out by SDS/polyacrylamide-gel (3.75% acrylamide) electrophoresis as previously described (Oike *et al.*, 1982). Protein was detected by staining with Coomassie Blue. The gel was then frozen and cut into 2 mm slices. The slices were each dissolved in 1 ml of Protosol and their radioactivity was measured in 10 ml of Aquasol-2.

Enzyme treatment

A sample of 1.5 mg (as protein) of PG(-CS,KS), to which $3.6 \times 10^5\text{ d.p.m.}$ of [^3H]mannose-labelled PG(-CS,KS) had been added as an internal marker, was dissolved in $80\text{ }\mu\text{l}$ of 0.01 M-HCl , and placed in a water bath at 90°C for 1 h. [Sialic acid residues, if any, should be eliminated by this procedure. It has been shown that asialoglycopeptides are preferred to the parent sialoglycopeptides as substrates for *N*-oligosaccharide glycopeptidase (Nishibe & Takahashi, 1981)]. A small amount of toluene and $40\text{ }\mu\text{g}$ of pepsin were added, and the mixture was incubated at 37°C for 48 h. The digest was evaporated to dryness under a reduced pressure. Hydrolysis with *N*-oligosaccharide glycopeptidase was carried out at 37°C for 20 h with concentrations of $920\text{ }\mu\text{g}$ (as protein) of pepsin hydrolysate product and 0.2 munit of enzyme/ $80\text{ }\mu\text{l}$ of $1\text{ M-citric acid/Na}_2\text{HPO}_4$, pH 5.0. The digest was centrifuged at $1500g$ for 20 min, and the supernatant was fractionated on a column ($0.65\text{ cm} \times 90\text{ cm}$) of Bio-Gel P-4, which was eluted with water at 53°C . Effluent fractions of volume about 1 ml were collected. Mannose-containing compounds were located by measuring the radioactivity of $20\text{ }\mu\text{l}$ samples of the effluents.

Hydrolysis with Pronase was carried out at 37°C for 48 h with concentrations of $400\text{ }\mu\text{g}$ (as protein) of substrate and $8\text{ }\mu\text{g}$ of enzyme/ $20\text{ }\mu\text{l}$ of 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.01 M-CaCl_2 . The reaction was terminated by placing the tube in a boiling-water bath for 5 min.

Hydrolysis with crystalline papain was carried out at 60°C for 15 h with concentrations of $400\text{ }\mu\text{g}$ (as protein) of substrate and $40\text{ }\mu\text{g}$ of enzyme/ $400\text{ }\mu\text{l}$ of $0.1\text{ M-sodium phosphate buffer}$, pH 6.5, containing 0.01 M-cysteine and 0.01 M-EDTA .

Thin-layer chromatography

About $5\text{ }\mu\text{l}$ of sample ($1\text{--}15\text{ }\mu\text{g}$ of carbohydrate or $10000\text{--}50000\text{ d.p.m.}$ of ^3H label) was applied to $10\text{ cm} \times 20\text{ cm}$ silica gel 60 plates. Samples were desalted beforehand by passing them through small columns ($0.4\text{ cm} \times 4\text{ cm}$) of Amberlite IR-120 (H^+ form) and Amberlite IRA-400 (CO_3^{2-} form) washed with water. Spots of the reference standards (isomalto-oligosaccharides, high-mannose-type oligosaccharides and hybrid-type oligosaccharides) were included on each plate. The plates were developed for about 20 h with propanol/acetic acid/water (3:3:2, by vol.) (Holmes & O'Brien, 1979). After chromatography, the plates were thoroughly dried. Radioactive compounds were detected by radioautography. Standard (unlabelled) carbohydrates were located by spraying the plates with 1% (w/v) orcinol in 50% (v/v) H_2SO_4 and heating them at 100°C for 10–20 min (Holmes & O'Brien, 1979).

Methylation analysis

Oligosaccharide samples on a thin-layer plate (located by radioautography; see above) were recovered by scraping radioactive zones from the plate and eluting the compounds with water. The samples were reduced and methylated by the method of Hakomori (1964). The permethylated products were extracted with chloroform and purified on a silica-gel column ($0.58\text{ cm} \times 5\text{ cm}$) as described by Endo *et al.* (1979). The purified samples were hydrolysed, reduced and acetylated as described by Stellner *et al.* (1973). The resulting alditol acetates were analysed with a Hewlett-Packard 5710A gas chromatograph combined with a JMS D-300 mass spectrometer, with a G-SCOT column ($0.28\text{ mm} \times 30\text{ m}$) of silicone OV-101; the injection temperature was 250°C , and the column temperature was from 150°C to 260°C at 3°C/min . Conditions for the mass spectra were: ionizing energy, 22 eV ; separator temperature, 220°C ; and accelerating voltage, 3 kV .

Other analytical procedures

Amino acids were determined by using a Hitachi 835 automatic amino acid analyser after hydrolysis of samples in a vacuum in $6\text{ M-HCl}/0.2\%$ (v/v) phenol at 110°C for 20 h. The total amount of cysteine and cystine was determined as cysteic acid by using the amino acid analyser after oxidation of samples with performic acid (Moore, 1963) and

subsequent hydrolysis, as described above. Tryptophan was determined by the procedure of Hugli & Moore (1972). Glucosamine and galactosamine were determined by using the amino acid analyser after hydrolysis of samples with 4M-HCl at 100°C for 4h. The neutral sugar composition of PG(−CS,KS) was determined by chromatography on a Shimazu LC-3A high-performance liquid chromatograph after hydrolysis with 2.5M-trifluoroacetic acid at 100°C for 6h. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Fraction V) as a standard. Hexuronate was determined by the carbazole method (Dische, 1947), with glucuronolactone as a standard.

Results

Chemical composition of PG(−CS,KS)

Table 2 shows the chemical composition of PG(−CS,KS). Protein accounts for 68% of the preparation (based on the Lowry assay; Lowry *et al.*, 1951) and its amino acid composition differs greatly from that for type Lb and Lt proteoglycans

Table 2. *Composition of PG(−CS, KS)*

Component	Amount ($\mu\text{mol}/100\text{mg}$ of core protein)
Amino acid	
Asx	46
Thr	77
Ser	93
Glx	130
Pro	68
Gly	86
Ala	64
CyS	3
Val	46
Met	7
Ile	38
Leu	47
Tyr	17
Phe	30
Lys	13
His	13
Arg	28
Trp	6
Sugar	
Glucosamine	40
Galactosamine	17
Hexuronic acid	35
Mannose	17
Galactose	83
Xylose	33
Glucose	33*

* This is at least partly a contaminant from the gels used for purification of PG(−CS,KS).

in the same tissue (Shinomura *et al.*, 1983; Noro *et al.*, 1983). The preparation also contains at least six kinds of sugar component. Our previous studies (Oike *et al.*, 1980) showed that chondroitinase ACII removes all the sulphated *N*-acetylgalactosamine units on the chondroitin sulphate chain of PG-H, whereas keratanase leaves about 10% of the sulphated *N*-acetylglucosamine units on the keratan sulphate chain. The equimolar amounts of xylose and hexuronic acid (Table 2) define the structure of the chondroitin sulphate remnant, $\Delta\text{GlcA-Gal-Gal-Xyl}$, and leave all the galactosamine of PG(−CS,KS) for the linkage region of protein-keratan sulphate remnant, $[\text{GlcNAc}(\text{SO}_4)\text{-Gal}]_n\text{-GalNAc}$, and protein-*O*-linked oligosaccharide (Lohmander *et al.*, 1980). Most, if not all, of the xylose, galactose, galactosamine and hexuronic acid of PG(−CS,KS) can be assigned to chondroitin sulphate remnants, keratan sulphate remnants and *O*-linked oligosaccharides. The mannose residues, however, cannot be assigned to these remnants.

When 1.2g of cartilages was incubated with $167\mu\text{Ci}$ of $[2\text{-}^3\text{H}]\text{mannose}$, the label was incorporated linearly into cartilages for at least 5h. From the tissues labelled for 5h, a PG-H sample with a specific radioactivity of $1.42 \times 10^6 \text{ d.p.m./mg}$ of protein was obtained. Treatment of the PG-H sample with chondroitinase ACII and keratanase yielded a protein-enriched core fraction, PG(−CS,KS), with a specific radioactivity of $1.0 \times 10^6 \text{ d.p.m./mg}$ of protein. Upon SDS/polyacrylamide-gel electrophoresis, the label co-migrated with the Coomassie Blue-staining material (Fig. 1), consistent with the prediction that some mannose-containing oligosaccharides are linked to the core protein. In the following experiments, advantage was taken of the fact that the mannose-containing moiety can be readily monitored by radioactivity when the labelled compound was used as an internal standard.

Digestion with N-oligosaccharide glycopeptidase

A purified preparation of PG(−CS,KS) was combined with $[^3\text{H}]\text{mannose}$ -labelled PG(−CS,KS) to give a specific radioactivity of $1.94 \times 10^5 \text{ d.p.m./mg}$ of protein, and the sample was treated sequentially with 0.01M-HCl, pepsin and *N*-oligosaccharide glycopeptidase. The final digest was chromatographed on Bio-Gel P-4 (Fig. 2). The enzyme digestion resulted in the appearance of a radioactive zone that was eluted like oligosaccharides (zone II). About 60% of the applied radioactivity was eluted in this zone. The remainder, however, was eluted in the excluded-volume zone (zone I).

Further treatment with Pronase or papain rendered only 2–5% of the zone-I material suscep-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of $[2-^3\text{H}]\text{mannose-labelled PG}(-\text{CS,KS})$

The gels in (a) and (b) are the same gel either stained with Coomassie Blue (a) or subjected to radioactivity scanning (b). The arrows indicate the positions of the following M_r markers: pig thyroid thyroglobulin subunit (M_r 330000), horse spleen ferritin (M_r 220000) and bovine serum albumin dimer (M_r 136000) and monomer (M_r 68000).

ible to *N*-oligosaccharide glycopeptidase, as shown in the inset in Fig. 2. When analysed by Bio-Gel P-30 chromatography, the zone-I material was eluted as a broad band corresponding in K_d value to proteins of M_r 10000–50000 (elution profile not shown). When examined by paper electrophoresis at pH 3.5, most of the zone-I material migrated towards the anode, whereas the zone-II material behaved as non-ionic compounds (electrophoretogram not shown). Thus the labelled material in zone I appears to be a group of large glycopeptides bearing acidic residues. Sugar analysis of zone-I material showed that the xylose/mannose ratio is about 1:1 and the galactosamine/mannose ratio is about 1.7:1. The former ratio is much lower than that for PG(-CS,KS) (Table 2). Conversely, the latter ratio is significantly higher than that for PG(-CS,KS). Since xylose and *N*-acetylgalactosamine may be regarded as the linkage sugar specific to chondroitin sulphate remnants and keratan sulphate remnants (or *O*-linked oligosaccharides) respectively (cf. Lohmander *et al.*, 1980),

Fig. 2. Elution profiles from Bio-Gel P-4 of products obtained after successive digestion of $[2-^3\text{H}]\text{mannose-labelled PG}(-\text{CS,KS})$ with pepsin and *N*-oligosaccharide glycopeptidase

V_0 indicates the void volume, and V_t the total column volume. G_4 and G_{10} indicate the positions for the isomalto-oligosaccharides of 4 and 10 glucose units respectively. Zones I and II were pooled as indicated by the horizontal bars and analysed as described in the text. When the zone-I material thus obtained was treated successively with Pronase and *N*-oligosaccharide glycopeptidase, the digest was chromatographed on the same column, the elution profile shown in the inset was obtained. Treatment with papain and *N*-oligosaccharide glycopeptidase also gave an identical result (not shown).

the difference between zone-I material and PG(-CS,KS) in the sugar ratio, coupled with the anionic nature of the zone-I material, suggests that the material in zone I was derived from a keratan sulphate-enriched region in PG(-CS,KS). Access of *N*-oligosaccharide glycopeptidase to the site of hydrolysis should be restricted when such an acidic glycosaminoglycan remnant is present in close proximity to *N*-oligosaccharide.

Purification and analysis of released oligosaccharides

The zone-II materials from the first and the second Bio-Gel P-4 columns (indicated by the bars in Fig. 2) were combined, desalted by passing them through ion-exchange resins, recovered by freeze-drying and dissolved in a small volume of water. A 50000 d.p.m. portion of the sample was chromatographed on a silica-gel thin-layer plate for oligosaccharide separation (Fig. 3). For comparison, a series of isomalto-oligosaccharides and a number of previously characterized oligosaccharides of the high-mannose type and the hybrid type were run on the same plate. It is evident that the chromatographic system has a high resolving power.

Fig. 3. Thin-layer chromatography of oligosaccharides (zone II in Fig. 2) released from [^3H]mannose-labelled PG(-CS,KS) by the glycopeptidase method

(i) Fluorogram of the chromatogram. For comparison, the chromatograms of reference oligosaccharides are shown in the right ordinates; (ii) isomalto-oligosaccharides of the indicated glucose (G) units; (iii) high-mannose-type and hybrid-type oligosaccharides A-J shown in Table 1. All these standard compounds were detected by staining with the orcinol/ H_2SO_4 reagent (Holmes & O'Brien, 1979).

The relative mobilities of the isomalto-oligosaccharides of 6–14 monosaccharide units when plotted graphically provide a curvilinear sequence of increasing mobilities with decreasing number of glucose units (Fig. 4). High-mannose-type oligosaccharides, $\text{Man}_{5-9}\text{GlcNAc}_2$, provide a significantly lower line. Hybrid-type oligosaccharides, $\text{Gal}_{0-1}\text{Man}_{4-5}\text{GlcNAc}_{4-5}$, are not on these lines. Their positions would predict that

oligosaccharides containing galactose, or *N*-acetylglucosamine, or both, in place of mannose (i.e. oligosaccharides of the hybrid or complex type) would be positioned in this representation on separate lines lying below that of the high-mannose-type oligosaccharides.

At least four radioactive compounds with chromatographic mobilities characteristic of oligosaccharides of the high-mannose type or

hybrid type were obtained from the zone-II material and designated IIa–IIc in the order of mobility. These compounds were eluted from the plate with water: the radioactivity proportions IIa:IIb:IIc:IIc were 0.6:1.0:0.3:0.1.

The thin-layer chromatography system was used on a preparative scale (with about 200 μ g of the zone-II material) to purify these compounds for further analyses. The following information about the four compounds was obtained.

Compound IIa. This compound had a chromatographic mobility identical with that of the high-mannose-type oligosaccharide $\text{Man}_7\text{GlcNAc}_2$ (Fig. 3). Methylation and analysis by gas chromatography–mass spectroscopy (Table 3) indicated that the compound contains non-reducing terminal (unsubstituted) mannose, 2-*O*-substituted mannose and 3,6-di-*O*-substituted mannose in the proportions 3:2:2 relative to 1 chitobiose unit. No methylated derivative indicative of a non-reducing terminal galactose or *N*-acetylglucosamine was detected. The results are consistent with the structure:

Definite proof is not yet available on the structure, but the known pathway of biosynthesis of *N*-linked oligosaccharides (for review see Schachter *et al.*, 1983) suggests that the above structure is the most likely possible one.

Compound IIb. The chromatographic mobility of this component is consistent with its represent-

ing the hybrid-type oligosaccharide $\text{Man}_5\text{GlcNAc}_4$ (Fig. 3). The profile of methylated derivatives (Table 3) showed that the compound contains unsubstituted mannose, 2-*O*-substituted mannose, 3,6-di-*O*-substituted mannose and 3,4,6-tri-*O*-substituted mannose in the proportions 2:1:1:1, relative to 1 chitobiose unit. Two molar equivalents of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol acetate indicative of two non-reducing terminal *N*-acetylglucosamine units were also detected. In view of the presence of a 3,4,6-tri-*O*-substituted mannose residue, one of these *N*-acetylglucosamine derivatives may be taken to indicate the presence of a bisecting *N*-acetylglucosamine residue. Among the previously found structures of this class of oligosaccharides, the following structure is consistent with the analytical results:

Compound IIc. This component occupied a position on the thin-layer chromatogram (Fig. 3) that suggests that it has a structure analogous to that of hybrid-type oligosaccharide G, $\text{Man}_4\text{GlcNAc}_5$, or H, $\text{Man}_5\text{GlcNAc}_5$. Consistent with this supposition, no methylated derivative indicative of a non-reducing terminal galactose residue was

Table 3. Methylation analysis of oligosaccharides IIa and IIb

Methylated derivative	Molar ratio	
	IIa*	IIb†
Galactitol		
2,3,4,6-Tetra- <i>O</i> -methyl	Trace‡	Trace‡
Mannitol		
2,3,4,6-Tetra- <i>O</i> -methyl	3.3	2.2
3,4,6-Tri- <i>O</i> -methyl	2.0	1.0
2,4-Di- <i>O</i> -methyl	2.1	0.9
2-Mono- <i>O</i> -methyl	0.0	1.1
2- <i>N</i> -Methylacetamido-2-deoxyglucitol		
1,3,5,6-Tetra- <i>O</i> -methyl	0.8	0.9
3,4,6-Tri- <i>O</i> -methyl	0.0	2.1
3,6-Di- <i>O</i> -methyl	1.1	1.1

* 3,4,6-Tri-*O*-methylmannitol set to 2.0.

† 3,4,6-Tri-*O*-methylmannitol set to 1.0.

‡ Less than 0.1.

detected (results not shown). Although methylation analysis indicated the presence of unsubstituted and substituted mannose and *N*-acetylglucosamine residues, it was difficult to obtain reproducible recoveries of the methylated derivatives, perhaps owing to a low degree of homogeneity of the sample.

Compound IID. Although this was present in an amount too small for quantitative methylation analysis, it was shown that this compound gave no tetramethylgalactitol acetate indicative of a non-reducing terminal galactose residue. Therefore this compound could not be a complex-type oligosaccharide. From the chromatographic profile (Fig. 3), it is suggested that this component represents a hybrid-type oligosaccharide analogous in structure to standard oligosaccharide I, Gal₁Man₄GlcNAc₅, or J, Gal₁Man₅GlcNAc₅.

Discussion

It is apparent from the experiments described in this paper that PG-H, the predominant proteoglycan of chick-embryo cartilage, contains at least four kinds of *N*-glycosidically linked oligosaccharide chains. PG(-CS,KS) migrates on SDS/polyacrylamide-gel electrophoresis as a protein with an apparent *M_r* of approx. 400 000 (Fig. 1). As its protein content, on the basis of the Lowry assay, is about 68%, it may be calculated that the *M_r* of the core protein is about 270 000. Since PG(-CS,KS) contains 17 μmol of mannose/100 mg of protein (Table 1), the average number of *N*-linked oligosaccharide chains on the core protein would be nine, on the assumption that the oligosaccharides contain five mannose residues/chain.

The structures for the predominant *N*-linked oligosaccharides are high-mannose and hybrid forms found on various glycoproteins such as ovalbumin (Tai *et al.*, 1975, 1977; Yamashita *et al.*, 1978) and are quite different from the structure of the predominant *N*-linked oligosaccharide in proteoglycan from the Swarm rat chondrosarcoma (Nilsson *et al.*, 1982):

The same complex-type oligosaccharide chain has also been found in proteodermatan sulphate from newborn-calf skin (Shinkai *et al.*, 1983). Since the present studies were limited to the glycopeptidase-releasable oligosaccharides, it is uncertain whether

Fig. 4. Plot of chromatographic mobility relative to isomalto-hexaose versus number of monosaccharide units for the oligosaccharides shown in Fig. 3

The positions of the isomalto-oligosaccharides (G₆-G₁₄) are indicated by ○ symbols, of the high-mannose-type oligosaccharides (A-E) by ● symbols, and of the hybrid-type oligosaccharides (F-J) by ▲ symbols. For the structure of Compounds A-J see Table 1.

or not PG(-CS,KS) contains the same or similar complex forms, although one may presume on the basis of the studies by De Luca *et al.* (1980) that some sialylated mannose-oligosaccharides of the complex type are contained in the glycopeptidase-resistant fraction.

In the present study, we utilized *N*-oligosaccharide glycopeptidase to release *N*-linked

oligosaccharide chains from the peptides. The enzyme cleaves β-aspartyl-*N*-acetylglucosylamine linkages in glycopeptides, releasing a carbohydrate chain with chitobiose at the free reducing end and leaving behind a carbohydrate-free peptide (Taka-

hashi & Nishibe, 1978). Non-sialylated and mono-sialylated complex-type oligosaccharides, as well as high-mannose and hybrid forms, were demonstrated from the hydrolysis of a variety of glycopeptides (Ishihara *et al.*, 1979; Plummer & Tarentino, 1981; Ishihara *et al.*, 1983), suggesting that the enzyme has a broad specificity with respect to the carbohydrate moiety. On the other hand, the effect of the nature of the peptide moiety seems to be related to its size and shape. For example, only 38% of the carbohydrate was removed from desialylated fibrinogen and its β - and γ -polypeptide chains, whereas over 95% of the oligosaccharides were released from the corresponding pepsin-released glycopeptides (Nishibe & Takahashi, 1981). In the native state, the oligosaccharides of ribonuclease B and the Fab_μ fragment derived from immunoglobulin M were completely resistant to the enzyme, but either heat denaturation in SDS or perturbation with chaotropic salts rendered these glycoproteins susceptible to the enzyme (Tarentino & Plummer, 1982). In view of these enzyme properties, we expected that digestion of PG(-CS,KS) with this enzyme may release all the *N*-glycosidically linked oligosaccharides, yielding an *N*-oligosaccharide-free core molecule. This approach, however, has led to the unexpected finding that a large proportion of the [³H]mannose-labelled moiety was resistant to *N*-oligosaccharide glycopeptidase even after degradation of the protein moiety by proteinases (Fig. 2). Some of the ³H radioactivity might represent sugar other than mannose, because the batch of [2-³H]mannose might have contained small amounts of ³H-labelled glucose or of ³H radioactivity on positions other than position 2 of the mannose, since the ³H would be lost if [2-³H]mannose were epimerized to glucose (De Luca *et al.*, 1980). The results of our preliminary analysis of the resistant fragments suggest that they are acidic glycopeptides of *M_r* values 10000–50000, in which glycosaminoglycan remnants (left by the action of chondroitinase ACII and keratanase) or *O*-linked oligosaccharides, or both, are located in the vicinity of *N*-linked oligosaccharides. Since the resistant glycopeptides are acidic and enriched in galactosamine relative to xylose, the mannose-containing oligosaccharides in the resistant fragments should represent those located in a region of the core protein where keratan sulphate chains are concentrated. The free oligosaccharides recovered in Zone II, in contrast, may be assigned to those located in a region of the core polypeptide with few or no attached chains of glycosaminoglycan and *O*-linked oligosaccharide. At any rate, the enzymic method was useful in liberating a class of oligosaccharides that raises the interesting possibility that another class of oligosaccharides, resistant to the

enzyme, resides on proteoglycans and requires experimental identification.

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