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An unsulphated region of the rat chondrosarcoma chondroitin sulphate chain and its binding to monoclonal antibody 3B3

John R. BAKER, James E. CHRISTNER and Suzanne L. EKBORG
Department of Biochemistry and the Atherosclerosis Research Unit, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

The chondroitin sulphate chains of proteoglycans are not uniformly sulphated. Commonly, regions of under- and over-sulphation are found. It is probable that variability in chondroitin sulphation has physiological significance, although such structure–function relationships largely remain unexplored. Chondroitin sulphate from rat chondrosarcoma proteoglycan has been found to possess no oversulphated residues. It is primarily chondroitin 4-sulphate, although a significant proportion of unsulphated disaccharides (14%) are also present. It appears that some unsulphated disaccharides are concentrated only at the point of attachment to the linkage region (i.e. it is the major unsaturated disaccharide remaining attached to chondrosarcoma proteoglycan core produced by chondroitinase ABC digestion). This proteoglycan core binds monoclonal antibody (MAb) 3B3. Although 3B3 principally binds to 6-sulphated 'stubs' of proteoglycan cores [Couchman, Caterson, Christner & Baker (1984) Nature (London) **307**, 650–652], given a high concentration of unsulphated 'stubs', it can alternatively bind to these residues. It is also evident that caution must be exercised in using MAb 3B3 to identify chondroitin 6-sulphated proteoglycans.

INTRODUCTION

The chondroitin sulphate (CS) chains of rat chondrosarcoma proteoglycan (RC PG) are 4-sulphated [2]. Some of the disaccharide repeat units were found to be unsulphated (13%), whereas none were 6-sulphated or oversulphated. There is no keratan sulphate or other type of glycosaminoglycan associated with this PG.

It is becoming increasingly apparent that the glycosaminoglycan chains of PGs are rarely, if ever, composed of homogeneous disaccharide repeats. Compositional heterogeneity may be introduced by lack of sulphation or oversulphation (i.e. ester sulphate substitution, which, in some regions, exceeds one per disaccharide repeat). In CS, regions of oversulphation due to 4,6-disulphation of *N*-acetylgalactosamine moieties or the presence of an additional ester sulphate on C-2 of some glucuronate moieties, which yield ΔDi-diS$_E$ and ΔDi-diS$_D$ after chondroitinase digestion respectively, have been reported [1]. The functional significance of this kind of heterogeneity is still largely unexplored, although it has been suggested that oversulphation of mast-cell CS PG may serve the purpose of binding proteinases [3]. Oversulphated regions of a PG's CS or dermatan sulphate chains may be involved in low-density-lipoprotein binding [4]. Thus RC PG, which is believed to possess no oversulphated regions, does not bind low-density lipoprotein [4]. The binding of some monoclonal antibodies (MAbs) to PGs also relates to the specific sulphation pattern of their constituent glycosaminoglycans [5,6].

The CS PG from RC is a commonly employed 'model' for studies of PG structure, specific binding and biosynthesis. In the present study we have verified that the PG is not oversulphated and is partially unsulphated. We have further shown that some unsulphated residues are concentrated close to the linkage region of this CS and appear to be responsible for binding MAb 3B3.

METHODS

Materials

Chodroitinases AC and ABC were from Sigma (St. Louis, MO, U.S.A.). Ammonium formate (Fisher; certified grade), water (Fisher; h.p.l.c. grade) and methanol (EM; Omnisolv grade) were used in the h.p.l.c. work. Nitrocellulose membranes (BA 85; 0.45 μm pore size) were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). A Lichrosorb Si 100 NH_2 column (250 mm × 4.0 mm; 5 μm particle size) and two Lichrosorb diol (250 mm × 4.0 mm; 10 μm particle size) columns were purchased from Alltech (Deerfield, IL, U.S.A.). The standard disaccharides ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS$_D$ and ΔDi-diS$_E$ were purchased from Seikagaku America (St. Petersburg, FL, U.S.A.). A PG monomer preparation was isolated from RC as described previously [7]. Mouse MAbs 1B5, 2B6 and 3B3 were previously raised and characterized in this laboratory [5]. MAb 3B3 in ascites fluid was further purified by precipitation with $(NH_4)_2SO_4$ and chromatography on Sephadex G-200 as previously described for another IgM MAb [8]. The purified MAb 3B3 was dissolved in Tris-buffered saline (0.02 M-Tris/0.5 M-NaCl, pH 7.5)/0.2% NaN_3 (TBS-azide) at approx. 1 mg/ml and stored in portions at −70 °C. MAbs 1B5 and 2B6 in ascites fluids were stored similarly.

Binding of MAbs

A qualitative dot-blot assay was employed. PG and PG protein-core samples were spotted on to nitrocellulose membrane and allowed to air-dry for 30 min. Subsequent blocking, exposure to first and second antibodies and colour development followed the protocol of Burnette [9]. First antibodies, MAbs 1B5, 2B6 and 3B3, were used at approx. 1 μg/ml in 1% gelatin/TBS-azide. The second antibody (horseradish peroxidase-conjugated goat anti-mouse IgG for 1B5 and 2B6) and chromogen (4-chloro-1-naphthol) were obtained as a kit (Bio-Rad) and used according

Abbreviations used: CS, chondroitin sulphate; RC PG, rat chondrosarcoma proteoglycan; MAb, monoclonal antibody; TBS, 0.5 M-NaCl/0.02 M-Tris/HCl, pH 7.5; DP, degree of polymerization; the abbreviations for unsaturated disaccharides released from chondroitin sulphate by chondroitinase action, ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS$_D$ and ΔDi-diS$_E$, are as given in [1].

to the manufacturer's instructions. To identify bound 3B3, which is an IgM, peroxidase-conjugated goat anti-mouse IgM from Fisher, Pittsburg, PA, U.S.A.), was substituted as second antibody.

Digestion of RC PG using chondroitinase AC or ABC

RC PG (0.5 mg) was dissolved in 0.1 M-Tris/HCl/0.03 M-sodium acetate, pH 7.4 (1.0 ml), chondroitinase AC (2 μl, 10 units/ml) was added, and the mixture was incubated at 20 °C for 24 h. The digestion was stopped by heating at 100 °C for 5 min, and the digest stored at −20 °C until analysed for oligosaccharides. Digestions with chondroitinase ABC were performed similarly, but at pH 8.0. To remove liberated oligosaccharides and recover the PG core, digests were dialysed exhaustively against water at 4 °C and freeze-dried.

Separation and quantification of oligosaccharides by h.p.l.c.

The h.p.l.c. system employed was from Waters Associates (Division of Millipore) and consisted of a model-712 Wisp autoinjector, a model-600 multisolvent delivery system, model-490 multiwavelength detector and model-820 Maxima data station.

Unsulphated and monosulphated disaccharides in chondroitinase AC or ABC digests of RC PG were fractionated isocratically on the Lichrosorb NH_2 column in aq. 0.30 M-ammonium formate/methanol (9:1, v/v) at a flow rate of 0.6 ml/min. The disulphated disaccharides were resolved isocratically in aq. 0.90 M-NaCl/0.01 M-sodium acetate, pH 6.5: methanol (9:1, v/v) on the same column and at the same flow rate. Disaccharides were monitored at 232 nm, identified and quantified from integrated peaks.

Analytical methods

The uronate contents of PG and PG protein-core preparations were determined by using a carbazole method [10]. A simple modification (addition of aq. Br_2 to the sample to a final concentration of 0.003%) prevented formation of colour and interference by unsaturated uronate residues. Unsaturated uronate residues of PG and PG protein-core preparations were selectively assayed using periodate/thiobarbituric acid [11].

RESULTS AND DISCUSSION

In the course of other studies, it was noted that RC PG which had been digested with chondroitinase ABC [i.e. RC PG(ABCase) core] binds MAb 3B3 (Fig. 1). However, RC PG is believed to possess no 6-sulphated residues [1], and the specificity of MAb 3B3 is for ΔDi-6S disaccharide residues at non-reducing termini of oligosaccharide 'stubs' on a proteoglycan protein core [12]. By contrast, the binding to RC PG(ABCase) core of MAbs 1B5 and 2B6 (Fig. 1), with specificities for non-reducing terminal ΔDi-0S and ΔDi-4S respectively, were predictable. We considered it important to investigate why 3B3 binds to RC PG(ABCase) core for two reasons. The specificity of MAb 3B3 may not be as limited as presently believed, and/or the CS chains of RC PG may contain a few, critical, 6-sulphated residues. Therefore we initially exhaustively digested RC PG with chondroitinase AC, under conditions described in the Methods section, and analysed by h.p.l.c. (Fig. 2) for the unsaturated disaccharides produced. Clearly, RC PG contains largely 4-sulphated residues and a significant proportion of unsulphated disaccharides (13–14%), but no 6-sulphated disaccharide (Fig. 2*c*). No disulphated disaccharides are present (Fig. 2*d*). In previous work [2], a similar conclusion was based upon separation of disaccharides by paper chromatography, a procedure less sensitive and more difficult to quantify than that used in the present work. Our findings clearly allow one to rule out the possibilities that 6-sulphated or oversulphated residues are responsible for the MAb 3B3 binding of RC PG.

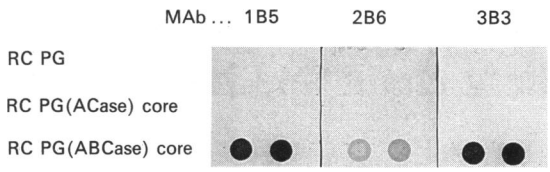

Fig. 1. Immunodot assay using MAbs 1B5, 2B6, and 3B3

RC PG, RC PG(ABCase) core and RC PG(ACase) core, each at 0.5 μg and 2.5 μg/50 μl of chondroitinase buffer, were applied to a nitrocellulose membrane with the aid of a Minifold apparatus (Schleicher and Schuell) before exposure to MAbs 1B5 or 3B3. (It was necessary to apply 12.5 μg and 25 μg/50 μl in order to reveal binding to 2B6.) Subsequent details of the procedure are given in the Methods section. In a control experiment, RC PG, labelled with ^{125}I through some tyrosine residues, was digested separately with chondroitinase AC and ABC. The PG, PG(ACase) and PG(ABCase) cores at 5 μg/50 μl in chondroitinase buffer bound efficiently (> 90%) to nitrocellulose.

After further digestion of RC PG(ABCase) core with chondroitinase AC, the resulting RC PG(ACase) core no longer bound MAb 3B3 (Fig. 1). Similarly, this PG core preparation lost its ability to bind 1B5 and 2B6. It was evident that the digestion with chondroitinase AC caused significant further removal of disaccharides from RC PG(ABCase) core (Table 1). Indeed, analyses indicated that very little CS disaccharide repeat structure remained as part of RC PG(ACase) core. Thus this core had a ratio of saturated to unsaturated uronate of 0.38, which means that 62% of this core's 'stubs' possess no disaccharide repeat units (i.e. no saturated uronate). The ratio of saturated to unsaturated uronate of RC PG(ABCase) core was 0.85, which indicates that it retains almost one disaccharide repeat per CS attachment site, and in this respect compares with the PG(ABCase) core from bovine nasal cartilage [11].

The disaccharides released by chondroitinase AC digestion of RC PG(ABCase) core were examined by h.p.l.c. and found to be enriched in ΔDi-0S [39.2 μg of ΔDi-0S and 19.6 μg of ΔDi-4S/mg of RC PG(ABCase) core; Table 1]. Thus, not only are there residual unsulphated disaccharide repeat units on the RC PG(ABCase) core, they are the principal kind of disaccharide present. This finding leads to the conclusion that 3B3 binding to RC PG(ABCase) core is via the latter's ΔDi-0S residues. At present it is uncertain whether a repeat disaccharide internal to a ΔDi-0S or ΔDi-6S is required for optimal binding of MAb 3B3. It is likely that MAb 3B3, applied to chondroitinase-treated tissue sections, will serve to immunolocalize both unsulphated and 6-sulphated CS chains of PGs. This antibody has been used widely for the recognition of chondroitin 6-sulphate PG in tissues (e.g. [13,14]), although data concerning its binding should be interpreted more cautiously.

That there is an unsulphated repeat sequence close to the linkage region of RC PG is not entirely unexpected. In thin sections of cartilage after digestion with chondroitinase ABC, PG was localized using MAb 1B5 [5], although PGs in similarly treated sections from other tissues could not bind this antibody. Also, the repeat disaccharide nearest the linkage region of chondroitin sulphate biosynthesized in embryonic-chick cartilage also appears to be unsulphated (T. B. Kulenschmidt, T. W. Chu, L. A. Bare & H. E. Conrad, unpublished work). At present we can only speculate on the reasons for this arrangement of unsulphated units in chondrosarcoma and cartilage PGs. One

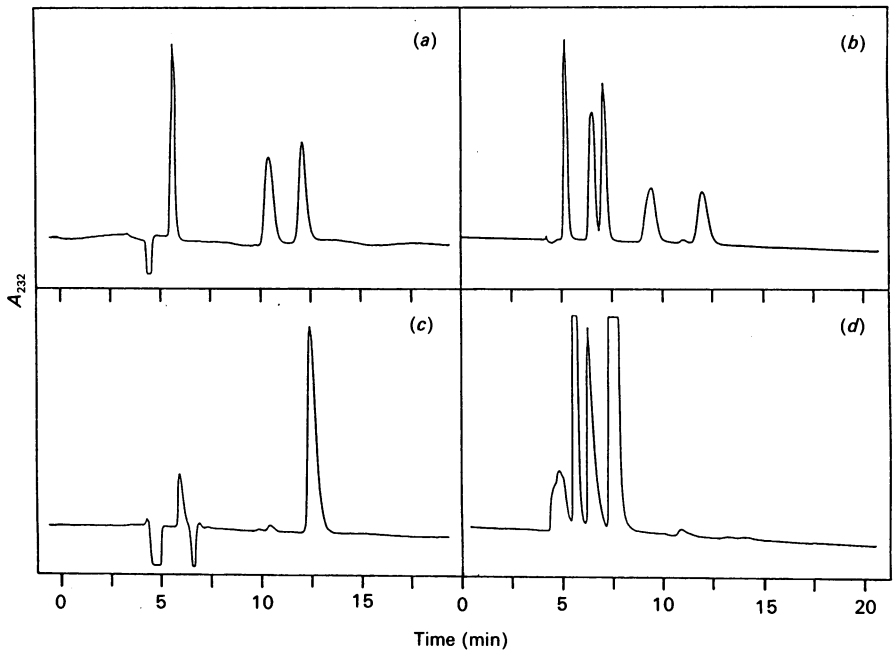

Fig. 2. Fractionation of disaccharides produced by chondroitinase AC digestion of RC PG

(*a*) Standard disaccharides (2.5 μg of each in 30 μl of water) were resolved isocratically in the ammonium formate system described in the Methods section. ΔDi-0S, ΔDi-6S and ΔDi-4S were eluted at 5.90, 10.64 and 12.30 min respectively. (*b*) Standard disaccharides, including disulphated disaccharides (2.5 μg of each in 50 μl of water), were resolved isocratically in the NaCl system described in the Methods section. ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-diS_D and ΔDi-diS_E were eluted at 5.30, 6.62, 7.19, 9.51 and 11.97 min respectively. (*c*) An aliquot (23 μl) of a chondroitinase AC digest of RC PG (see the Methods section) was fractionated as in (*a*). Only ΔDi-0S (0.79 μg) and ΔDi-4S (5.67 μg) were identified and quantified. The small peak at approx. 10.5 min is not coincident with ΔDi-6S. (*d*) An aliquot (200 μl) of the same digest as in (*c*) was fractionated as in (*b*). No peaks corresponding to the disulphated disaccharides ΔDi-diS_D or ΔDi-diS_E were seen. The peak at 6.24 min (not seen in *b*) is due to acetate ion.

Table 1. Release of disaccharides upon digestion of RC PG(ABCase) core by chondroitinase AC

RC PG(ABCase) core (0.75 mg) in 0.1 M-Tris/HCl/0.03 M-sodium acetate, pH 7.4 (1.0 ml) was incubated with 0.02 unit of chondroitinase AC at 20 °C for 24 h and the course of the digestion was monitored by the increase in A_{232}. To ensure completeness of digestion, a further 0.02 unit of enzyme was added and the digestion prolonged for a further 24 h at 20 °C. Ethanol (4.0 ml) was added to precipitate PG(ACase) core, the supernatant evaporated to dryness to recover released disaccharides, which were dissolved in water (250 μl). An aliquot (25 μl) was fractionated by h.p.l.c., as in Figs. 2(*a*) and 2(*c*) to separate and quantify ΔDi-OS and ΔDi-4S. As a control, RC PG(ABCase) core was also similarly incubated with chondroitinase ABC at pH 8.0. In this case there was little further release of disaccharides.

	Disaccharide released (μg/mg of core)	
Chondroitinase	ΔDi-0S	ΔDi-4S
ABC	9.23	7.94
AC	38.8	19.5

possibility is that sulphotransferases have difficulty gaining access to sites near the protein core as the biosynthesis of closely spaced chondroitin chains commences. Also, the essential biological activity of the PG (e.g. in binding water) may be little affected by lack of sulphation of chains close to the protein core.

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