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A novel keratan sulphate proteoglycan from a human embryonal carcinoma cell line

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We describe here the purification and partial characterization of a 200 kDa keratan sulphate proteoglycan found in the pericellular matrix of human embryonal carcinoma cells. Previously we have shown that this molecule is recognized by a monoclonal antibody (GCTM-2). The antigen was isolated using ion-exchange chromatography and gel filtration, purification being monitored by e.l.i.s.a. using GCTM-2. Metabolic labelling of GCT 27 C-4 embryonal carcinoma cells with sodium [^{35}S]sulphate resulted in the incorporation of [^{35}S]sulphate into the purified molecule. Throughout the purification procedure, the peaks of ^{35}S radioactivity were coincident with the peaks of immunoreactivity, and this label was released both by digestion with keratanase and chondroitinase, confirming the proteoglycan nature of the antigen. The intact molecule ran as a single broad band of 200 kDa, which has been identified by silver staining and immunoblotting following gel electrophoresis. Amino acid analysis of the purified antigen indicated a high content of serine, glycine and aspartic acid/asparagine residues. Visualization by rotary-shadowing electron microscopy suggests that the purified material forms large aggregates, even under denaturing conditions. Deglycosylation of this preparation with trifluoromethanesulphonic acid yielded a major band of 55 kDa and a minor band of 48 kDa. The biochemical nature of the molecule described here, along with tissue distribution studies using GCTM-2, indicates that the antigen is not related to previously described keratan sulphate proteoglycans.

INTRODUCTION

The growth, differentiation and movement of cells are to a large part regulated by their interactions with the extracellular matrix. Proteoglycans, comprising a protein core covalently linked to glycosaminoglycans, are abundant molecules found at the cell surface, in the extracellular matrix and in secretory granules. They are often large aggregating molecules which can interact with other matrix molecules, such as cell attachment factors, and with growth factors [1–4]. These interactions are mediated both by the highly charged glycosaminoglycan side-chains and through specialized domains of the protein core.

Proteoglycans have been characterized by cDNA cloning of their core proteins and by analysis of their glycosaminoglycan components. So far, keratan sulphate glycosaminoglycan chains have been found covalently attached to part of the large bovine cartilage proteoglycan [5,6] and to proteoglycans found in corneal stroma [7–10]. A collagen-binding proteoglycan, fibromodulin, with between one and four keratan sulphate chains attached to a 42 kDa core protein [11], and a corneal keratan sulphate proteoglycan, lumican [12], with a core protein of 38.6 kDa, have recently been cloned. The core-protein sequences of these molecules are related to those of the chondroitin sulphate/dermatan sulphate proteoglycan, decorin [13], and to that of the small bone proteoglycan, biglycan [14].

Previously [15], we have described a monoclonal antibody, GCTM-2, which recognizes a pericellular matrix antigen present on several human embryonal carcinoma cell-lines. Immunoblotting of embryonal carcinoma cell-line GCT 27 C-4 cell lysates revealed that this antibody recognized a 200 kDa molecule. Keratanase (from *Pseudomonas* sp.) treatment of intact cells before immunoblotting enhanced GCTM-2 binding to the antigen and degraded the molecule to a smaller immunoreactive band of 55 kDa. However, as reported previously [15], the molecular mass of the antigen was not altered by digestion with N-Glycanase, heparatin sulphate lyase or chondroitinase ABC.

Immunocytochemical staining of tissues with GCTM-2 [15a] indicated that the antigen has a limited distribution. Strongest staining was found in foetal gut epithelium and muscle. However, in cell lines representative of these tissues, the recognized epitope appeared to correspond to a smaller intracellular form of the molecule (50–70 kDa), which is resistant to keratanase degradation. GCTM-2 also stained a high proportion of the malignant human germ-cell tumours examined [15a].

Studies using a multipotent teratocarcinoma cell clone, GCT 27 X-1 [16], revealed that proteoglycan expression is differentiation-dependent: the antigen is only expressed on the surface of these embryonal carcinoma stem cells and is lost upon spontaneous differentiation *in vitro*, or following retinoic-acid-induced differentiation of the teratocarcinoma cell-line N-Tera-2 C-13 (M. F. Pera, unpublished work).

Preliminary studies [17] using GCTM-2 in a competitive e.l.i.s.a. suggested that the antigen may be a potential serum marker for patients with malignant testicular germ-cell tumours.

We describe here the isolation, together with partial biochemical and molecular characterization, of this novel keratan sulphate proteoglycan.

MATERIALS AND METHODS

Materials

The cell-line GCT 27 C-4, a nullipotent clone of human embryonal carcinoma cells, was cultured as described [16].

Alcian Blue was obtained from National Diagnostics, Aylesbury, Bucks. U.K. Heparan sulphate, heparatin sulphate lyase (heparatinase I; EC 4.2.2.8 from *Flavobacterium heparinum*),

Abbreviations used: TFMS, trifluoromethanesulphonic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulphonate; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; Blotto, 5% (w/v) non-fat dry milk; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor β.

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Tween 20, trifluoromethanesulphonic acid (TFMS), and pre-stained molecular-mass markers were from Sigma Chemical Co., Poole, Dorset, U.K. Antibodies to human fibronectin and laminin were purchased from Calbiochem (U.K.) Ltd., Nottingham, U.K., and antibody to type IV collagen from Serotec Ltd, Kidlington, Oxon, U.K. Anti-mouse immunoglobulin antibody coupled to biotin, streptavidin conjugated to horseradish peroxidase, chemiluminescent substrate (luminol) and sodium [^{35}S]sulphate (176–282 mCi/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. DEAE-Sepharose Fast Flow, Superose 6 HR 10/30 columns and PD10 Sephadex G-25 disposable columns were obtained from Pharmacia LKB, Milton Keynes, U.K. Collodion bags (molecular-mass cut-off 12400 Da) and holders were purchased from Sartorius Instruments, Epsom, Surrey, U.K. The substrate for e.l.i.s.a., 2,2′-azinodi[(3-ethylbenzothiazoline)-6-sulphonate (ABTS), was supplied by Boehringer–Mannheim, Lewes, Sussex, U.K. Keratanase (keratan sulphate 1,4-β-D-galactanohydrolase; EC 3.2.1.103; *Pseudomonas* sp.) and chondroitinase ABC (chondroitinase ABC lyase; EC 4.2.2.4; *Proteus vulgaris*) were obtained from ICN Biomedicals Ltd., High Wycombe, Bucks., U.K. Immobilon P immunoblotting membrane was supplied by Millipore (U.K.), Watford, Herts., U.K., and Quickzint 401 aqueous scintillation cocktail was purchased from Zinsser Analytic, Maidenhead, Berks., U.K. All other chemicals were of analytical grade.

Analytical methods

Protein content was determined using the method of Bradford [18]. Glycosaminoglycan content was estimated using the Alcian Blue binding method of Whiteman [19] with heparan sulphate at 6–100 μg/ml as a standard. [^{35}S]Sulphate radioactivity was measured using Quickzint 401 gel scintillant for aqueous solutions in an LKB 1215-Rackbeta liquid scintillation counter.

E.l.i.s.a.

Samples were serially diluted into 0.05 M-sodium bicarbonate buffer, pH 9.6, and incubated on microtitre plates overnight at 4 °C. The plates were rinsed with 0.1 % Tween 20 in phosphate-buffered saline (PBS) and vacant sites blocked by incubation for 1 h with 1 % BSA in PBS. GCTM-2 hybridoma supernatant was added without dilution; monoclonal antibodies to fibronectin, laminin and type-IV collagen were diluted 1:1000 in 1 % BSA. Primary antibodies were incubated in the wells for 1 h. Specific binding of antibody was detected using biotinylated sheep anti-mouse immunoglobulin diluted 1:500 in 1 % BSA for 1 h, followed by streptavidin coupled to horseradish peroxidase diluted 1:500 in 1 % BSA for 30 min. The reaction was quantified by adding ABTS (1 mM) with hydrogen peroxide (0.002 %, v/v) in 0.1 M-phosphate/citrate buffer, pH 4.3, as a substrate. Absorbance was measured at 414 nm in a Titertek Multiscan plate reader.

The results from the e.l.i.s.a. were analysed using the method of Ritchie *et al.* [20]. The sigmoidal titration curves were linearized using a logit–log transformation of the data. The maximum value was nominally given as 100 % and all other values were calculated relative to this.

Extraction and purification of proteoglycan

Confluent monolayers of GCT 27 C-4 cells were washed twice with PBS at 4 °C and then extracted with a solution containing 1 M-guanidine hydrochloride, 0.05 M-sodium acetate, 10 mM-EDTA, 5 mM-benzamidine hydrochloride and 1 mM-phenylmethanesulphonyl fluoride (PMSF) for 1 h at 4 °C. The extracts were clarified by centrifugation at 10000 *g* for 20 min at 4 °C. The remaining procedures were carried out at room temperature. Extracts were exchanged into 7 M-urea in 0.05 M-Tris/HCl, pH 6.8, using PD10 Sephadex G-25 columns. This material was applied to an HR 10/10 column containing 8 ml of DEAE-Sepharose Fast Flow and eluted with a linear gradient (100 ml) of 0–1 M-NaCl using f.p.l.c. at a flow rate of 1.5 ml/min. Fractions (2 ml) were taken and aliquots analysed for antibody binding with GCTM-2 using e.l.i.s.a. The immunoreactive fractions were concentrated by vacuum dialysis against 7 M-urea in 0.05 M-Tris/HCl, pH 6.8, using Sartorius collodion bags. The concentrated material was then separated on a Superose 6 HR 10/30 column (24 ml) which had previously been equilibrated with 2 M-guanidine hydrochloride/0.5 M-sodium acetate, pH 6.8. Fractions (0.5 ml) were collected and the immunoreactive fractions (as determined by e.l.i.s.a.) were pooled. Concentration of purified proteoglycan and stepwise dialysis from guanidine hydrochloride into PBS was achieved using Sartorius collodion bags.

Metabolic labelling of GCT 27 C-4 cells with [^{35}S]sulphate

GCT 27 C-4 cells were labelled for 18 h with [^{35}S]sulphate, (10 μCi/ml) in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal calf serum. Extraction and purification procedures were as described above. Throughout the purification, antigen content was monitored using e.l.i.s.a. and the incorporation of [^{35}S]sulphate radioactivity was measured by liquid scintillation spectroscopy.

Enzyme digestion

Purified [^{35}S]sulphate-labelled proteoglycan was dialysed against enzymic digestion buffer (according to the manufacturer's instructions) with 0.1 % BSA added as a carrier protein. Two units of keratanase, chondroitinase ABC or heparatin sulphate lyase were added and digestion conducted for 2 h at 37 °C (or 43 °C in the case of heparatin sulphate lyase). An equal volume of 4 M-guanidine hydrochloride/1 M-sodium acetate, pH 6.8, was added to the mixture which was then run through the Superose 6 column in 2 M-guanidine hydrochloride/0.5 M-sodium acetate, pH 6.8. Fractions (0.5 ml) were collected and [^{35}S]sulphate content measured. The radioactivity of each fraction was expressed as a percentage of the total number of recovered counts in each run.

Amino acid analysis

Amino acid analyses were carried out by Dr. Tony Willis, MRC Immunochemistry Unit, Oxford. Purified proteoglycan samples were hydrolysed in 5.7 M-HCl at 150 °C for 75 min. Following pre-column derivatization using phenylisothiocyanate, the hydrolysate was analysed on a Waters Pico Tag System from Millipore (U.K.) Ltd. Correction was made for hydrolytic losses of serine and threonine by increasing the determined values of serine by 20 % and those of threonine by 10 %. No determinations for cysteine or tryptophan residues were made. Although some glucosamine (GluN) and galactosamine (GalN) residues were detected in the hydrolysate, proper determinations for these amino sugars, corrected for hydrolysis, were not made.

Preparation of core protein

The core protein was prepared using TFMS hydrolysis in accordance with Edge *et al.* [21]. Deglycosylated protein was isolated by adding a 2-fold excess of diethyl ether cooled to −40 °C. An equal volume of ice-cold aqueous pyridine (50 % v/v) was added, and the resulting precipitate was redissolved by vortexing. The ether phase was removed and discarded. After a second diethyl ether extraction, the aqueous phase was dialysed

against water and lyophilized before undergoing gel electrophoresis.

PAGE and immunoblotting

Proteoglycan, or its core protein, was electrophoresed according to the procedure of Laemmli [22] on a Bio-Rad Mini Protean slab gel system. Staining was accomplished with silver [23] or periodic silver stain [24]. Prestained standards (26–180 kDa) were used as molecular-mass markers.

Electrophoretic transfer of antigens separated by SDS/PAGE on to Immobilon-P membrane was performed in 10 mM-3-(cyclohexylamino)-1-propanesulphonic acid (CAPS), pH 11, at 30 mV/cm for 18 h at 4 °C. After blotting, the filter was blocked for 1 h with 5% Blotto [5% (w/v) non-fat dry milk] in 0.1% Tween 20 in PBS. Incubation with GCTM-2 supernatant was performed for 1 h, followed by incubation with sheep anti-mouse immunoglobulin conjugated to biotin at a dilution of 1:200 for 1 hour, and then streptavidin conjugated to horseradish peroxidase at a dilution of 1:200 for 30 min. Chemiluminescent substrate (luminol) was used according to the manufacturer's instructions.

Electron microscopy

Purified intact proteoglycan samples in PBS were prepared for rotary shadowing essentially by the method of Tyler & Branton [25]. Equal volumes of sample (at 30 μg/ml) and glycerol were mixed and sprayed on to freshly cleaved mica, they were dried at room temperature under vacuum, rotary-shadowed with platinum at an angle of 6° and coated with carbon. The metal and carbon replica was floated off on to water and picked up on a copper–rhodium grid for examination under a Philips EM301 electron microscope at a nominal magnification of ×45000.

Samples in 2 M-guanidine hydrochloride were applied to carbon-coated grids, washed briefly with a few drops of water and then washed extensively by floating overnight in water. The grids were air-dried and rotary-shadowed with platinum at an angle of 6°. All observations and measurements were made at a total magnification of ×115000.

To study the interaction of the proteoglycan with the antibody GCTM-2, carbon-coated grids were positively charged by floating for 3 min on a 0.001% solution of benzyldimethylalkylammonium chloride. Proteoglycan was applied to the grids for 3 min; they were then rinsed briefly with a few drops of 0.1 M-KCl. The grids were then floated on a solution of diluted purified antibody for 30 min, rinsed with PBS and air-dried. They were then either stained with 1% (w/v) uranyl acetate or rotary-shadowed as described above.

Control experiments were also carried out using another mouse monoclonal IgM antibody, GCTM-4, which recognizes another, unrelated, intracellular molecule in GCT 27 C-4 cells [15].

RESULTS

Purification, PAGE and immunoblotting of proteoglycan

The steps in the isolation of proteoglycan from GCT 27 C-4 cells are summarized in Table 1. The initial extraction conditions were designed to remove a high proportion of extracellular proteins without causing cell lysis and subsequent contamination with intracellular proteins. A second extraction of the cellular pellet following centrifugation did not result in a significant removal of GCTM-2 antigen, suggesting that the primary extraction process resulted in the removal of 90% of the total immunoreactive material (results not shown). The peak containing the proteoglycan eluted from the DEAE-Sepharose column at 0.6 M-NaCl as determined by e.l.i.s.a. with GCTM-2

Table 1. Purification of keratan sulphate proteoglycan from embryonal carcinoma cells

Experimental details are given in the Materials and methods section.

Purification step	Protein content (μg)	Glycosaminoglycan content (μg)	Protein/glycosaminoglycan ratio	Purification (fold)
Guanidine extraction	1100	352	0.032	1
DEAE Sepharose chromatography	300	60	0.2	6.25
Superose 6 chromatography	10	7	0.7	22

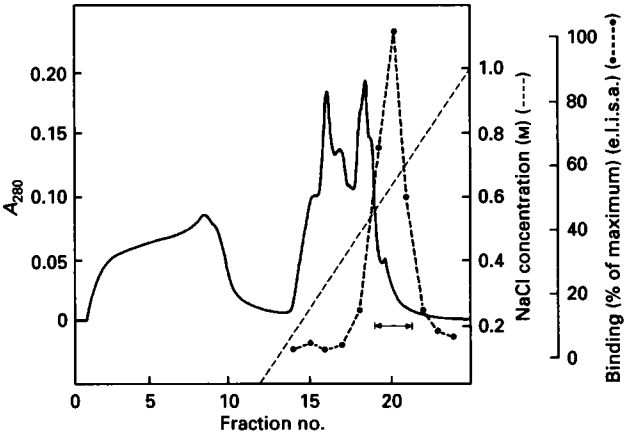

Fig. 1. Chromatography of GCT 27 C-4 extract on a DEAE-Sepharose CL-CB fast flow column

Elution was with 7 M-urea in 0.05 M-Tris/HCl, pH 6.8, followed by a gradient of 0–1 M-NaCl (------) in the same buffer. Protein was monitored by A_{280} (——) and antigen binding to GCTM-2 was determined by e.l.i.s.a. (●). Peak fractions were pooled as indicated for further purification.

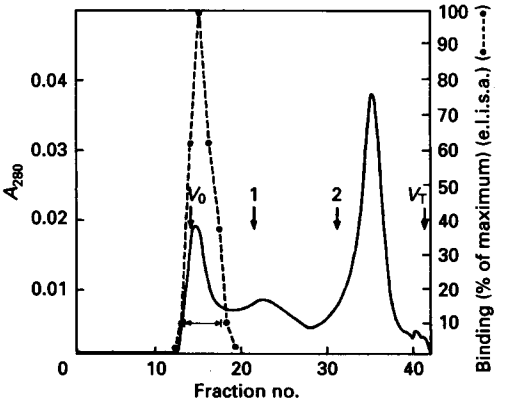

Fig. 2. Gel chromatography on Superose 6 of the peak fraction from DEAE-Sepharose chromatography

The column was equilibrated with 2 M-guanidine hydrochloride, 0.5 M-sodium acetate, pH 5.8. Protein was monitored by A_{280} (——) and the antigen binding to GCTM-2 was determined by e.l.i.s.a. (●). The proteoglycan eluted as a single peak just after the void volume (V_0). The total column volume (V_T) and elution positions of the molecular mass markers thyroglobulin, 669 kDa (1), and aldolase, 158 kDa (2), are indicated.

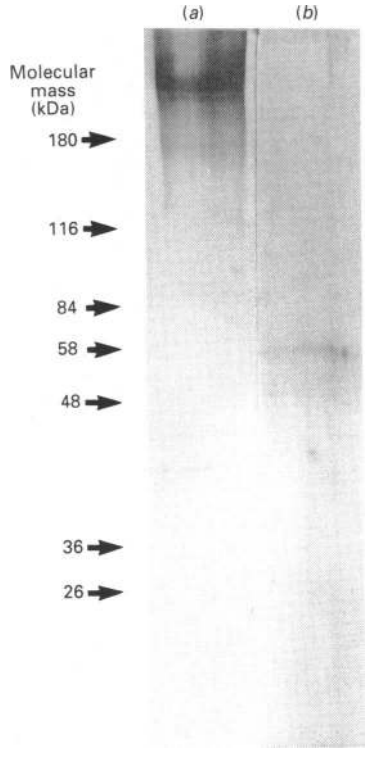

Fig. 3. SDS/PAGE (10%) of the purified proteoglycan before (*a*) and after (*b*) TFMS hydrolysis

Approx. 20 μg of intact proteoglycan (track *a*) was applied and the gel was stained with silver. Positions of the molecular mass markers are indicated.

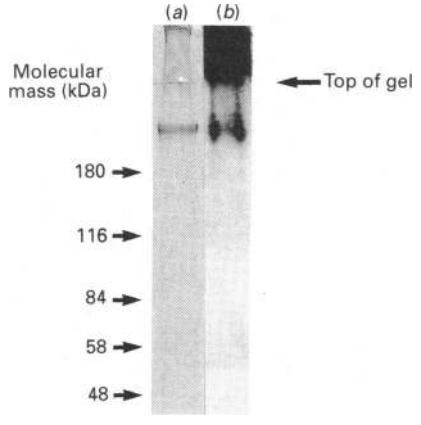

Fig. 4. SDS/PAGE (7.5%) of the purified proteoglycan after silver staining (*a*) and after immunoblotting with GCTM-2 (*b*)

Positions of the molecular mass markers are indicated.

(Fig. 1) and by Alcian Blue binding (results not shown). Superose 6 gel filtration results in the removal of lower-molecular-mass contaminants. The proteoglycan peak eluted as a sharp peak just after the void volume of the column (Fig. 2). The purified fraction was free from contamination with laminin, fibronectin and type IV collagen, as determined by e.l.i.s.a. (results not shown), and ran as a single broad band on 10% or 7.5% gel

Table 2. Amino acid composition of keratan sulphate proteoglycan

The GluN/GalN ratio was found to be 8:1.

Amino acid	Composition (residues/10^3 amino acid residues)
Aspartic acid + asparagine	80
Glutamic acid + glutamine	128
Serine	118
Glycine	172
Histidine	12
Arginine	44
Threonine	48
Alanine	64
Proline	44
Tyrosine	72
Valine	50
Methionine	46
Isoleucine	28
Leucine	62
Phenylalanine	22
Lysine	10

electrophoresis (see Figs. 3*a* and 4*a*). This band also immunoblotted with GCTM-2 (Fig. 4*b*) and corresponds to a molecular mass of approx. 200 kDa. In addition, a large amount of immunoreactive material which did not migrate into the main gel transferred from the stacking gel [3.75% (w/v) acrylamide] (see Fig. 4*b*). When purified proteoglycan was electrophoresed on a mixed acrylamide and agarose gel [26] electrophoretic transfer was more efficient than from pure acrylamide gels, but the immunoreactive material ran as an extended smear throughout the length of the gel rather than as a discrete band (S. Cooper, unpublished work).

If subconfluent cell monolayers were extracted, another peak of immunoreactivity eluted from the DEAE-Sepharose column at 0.25–0.3 M-NaCl. This material eluted as two immunoreactive peaks when subjected to Superose 6 gel filtration: one just after the void volume, as previously, and another at a lower molecular mass (approx. 700 kDa). The first peak on Superose immunoblotted at 200 kDa and the second at 200 kDa with a fainter band at 150 kDa, but both were devoid of any immunoreactive material in the stacking gel (S. Cooper, unpublished work). Since the DEAE-Sepharose 0.25–0.3 M-NaCl eluent was heavily contaminated with a large amount of lower-molecular-mass proteins, this material was not investigated further in this study.

Characterization of the proteoglycan

The proteoglycan fractions from Superose 6 gel filtration of the high-salt eluent from DEAE-Sepharose chromatography were pooled, concentrated and dialysed stepwise into PBS, using vacuum dialysis.

This material was subjected to amino acid analysis, the results of which are shown in Table 2. The amino acid composition is similar to that found for other proteoglycans [5,8,27,28] with large amounts of serine, glycine and glutamic acid (plus glutamine).

Quantitative measurements of GlcN and GalN were not made since large amounts will have been lost during hydrolysis. However, the GlcN/GalN ratio of 8:1 (w/w) is consistent with the major glycosaminoglycan being keratan sulphate [7]. The lack of hydroxyproline and hydroxylysine in the analysis confirms the absence of collagenous material in the preparation.

The gel-filtration profile obtained following antigen purification from GCT 27 C-4 cells metabolically labelled with

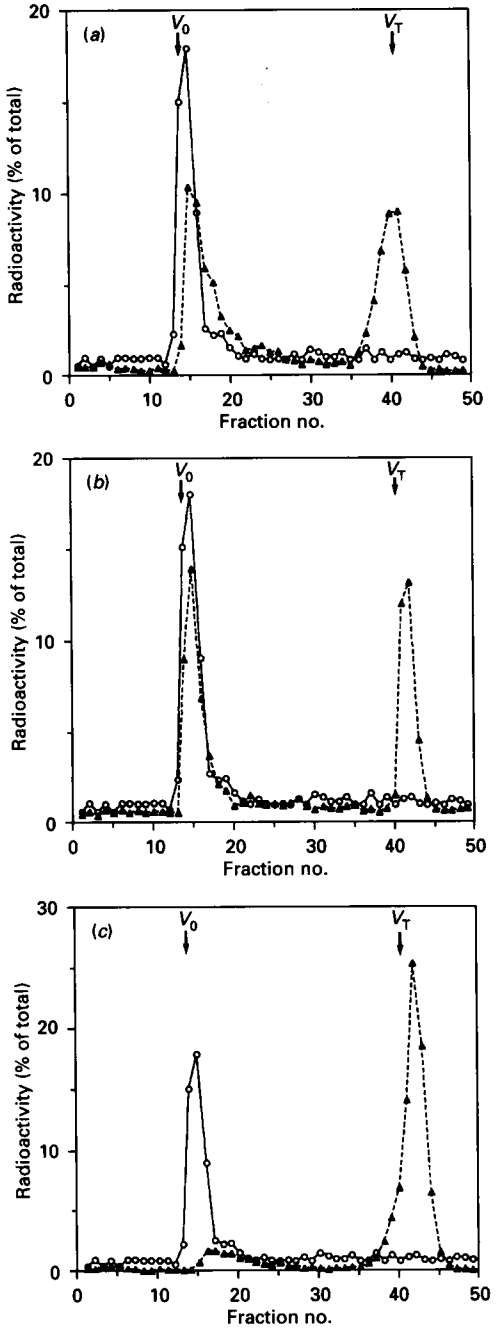

Fig. 5. Gel chromatography on Superose 6 of [^{35}S]sulphate-labelled purified proteoglycan before (○) and after (▲) digestion with (*a*) keratanase, (*b*) chondroitinase ABC, and (*c*) keratanase followed by chondroitinase ABC

The radioactivity of each fraction is expressed as a percentage of the total number of recovered counts in each run. Positions of the void volume (V_0), and total volume (V_T) are shown.

[^{35}S]sulphate is shown in Fig. 5. Before enzymic digestion, the peaks of [^{35}S]-sulphate (shown by the solid lines in Fig. 5*a–c*) and GCTM-2 binding, as determined by e.l.i.s.a. (M. F. Pera & S. Cooper, unpublished work) were coincident.

Repeated digestion of the purified material with keratanase released 46% of the total incorporated [^{35}S]sulphate (Fig. 5*a*). Chondroitinase released 41% (Fig. 5*b*). The released label ran as a low-molecular-mass form near the total volume of the column. Sequential digestion with keratanase followed by chondroitinase

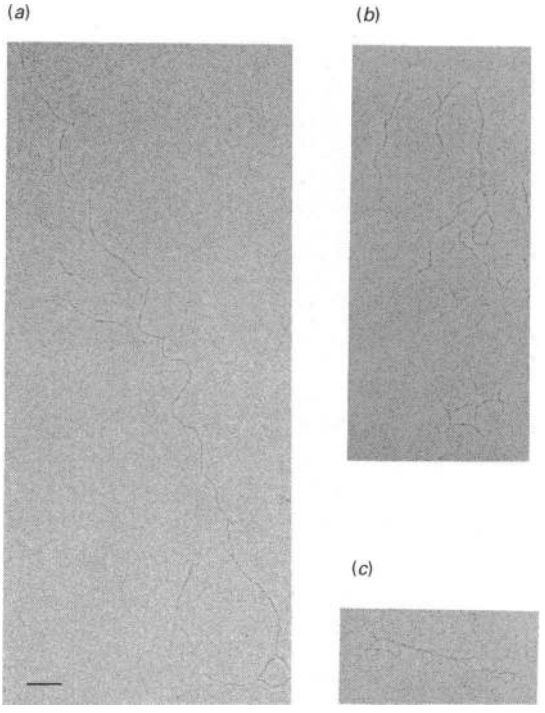

Fig. 6. Electron micrographs of rotary-shadowed proteoglycan preparations in PBS

(*a*) shows an aggregate of several microns in length and of variable width. (*b*) and (*c*) illustrate the side-by-side aggregation of these molecules. The bar represents 100 nm.

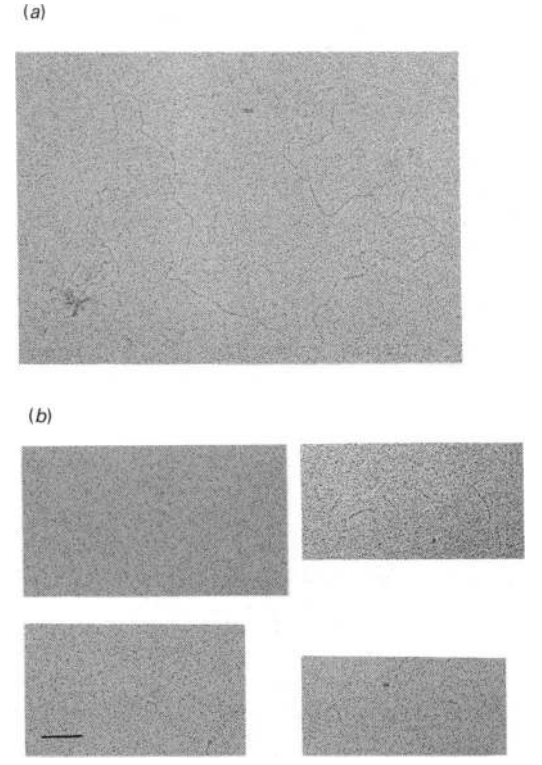

Fig. 7. Electron micrographs of rotary-shadowed proteoglycan preparations in 2 M-guanidine hydrochloride

(*a*) A single end-to-end aggregate of uniform width. (*b*) Smaller 80 ± 10 nm molecules of uniform width. The bar represents 100 nm.

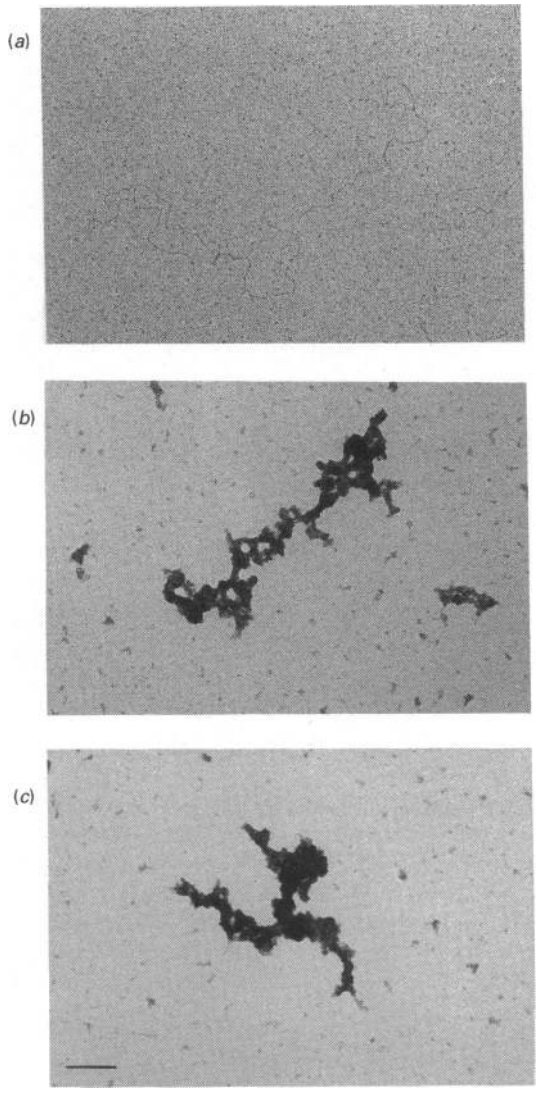

Fig. 8. Electron micrographs of proteoglycan samples incubated with GCTM-2 and then stained with uranyl acetate

(*a*) Antigen alone, (*b*) and (*c*) antigen with bound GCTM-2. The bar represents 100 nm.

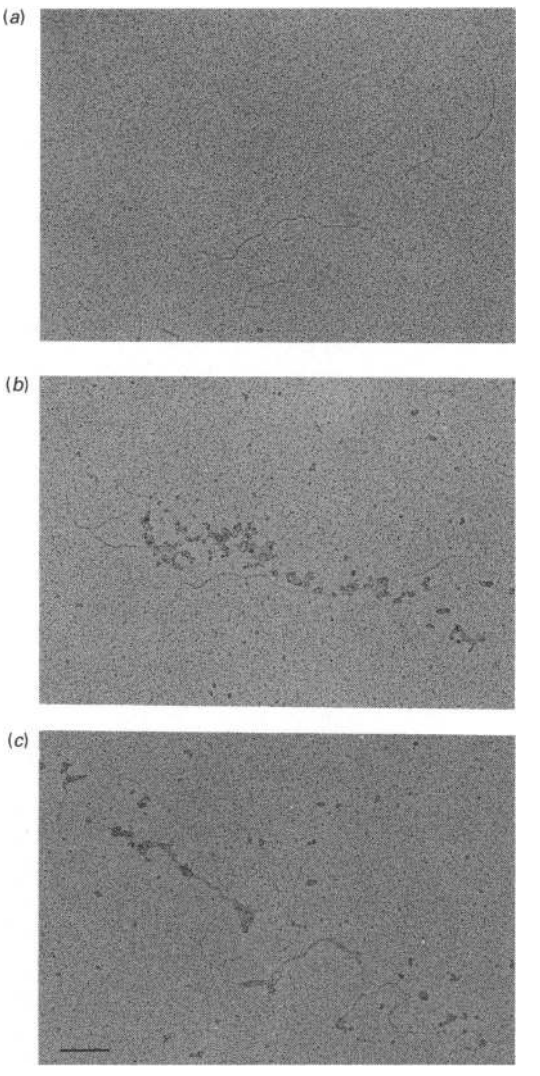

Fig. 9. Electron micrographs of proteoglycan samples incubated with GCTM-2 and then rotary-shadowed

(*a*) Antigen alone, (*b*) and (*c*) antigen with bound GCTM-2. The bar represents 100 nm.

released 87% of the incorporated [^{35}S]sulphate (Fig. 5*c*). The immunoreactive peak, containing the remaining 13% of [^{35}S]sulphate, ran just after the void volume during Superose 6 gel filtration ([^{35}S]sulphate results are shown by the dashed line in Fig. 5*c*).

Heparatin sulphate lyase did not release significant [^{35}S]sulphate.

Treatment of the purified proteoglycan with TFMS, a reagent that cleaves off all saccharide units, resulted in complete degradation of the 200 kDa band to one of 55 kDa. A fainter band of 48 kDa is also visible (Fig. 3*b*). There is a pronounced sharpening of the electrophoretic bands after TFMS treatment which suggests that the width of the 200 kDa band is mainly due to variability in glycosaminoglycan substitution of the polypeptide core. This result is in agreement with keratanase treatment of the proteoglycan [15] which also results in partial reduction of the 200 kDa band to 55 kDa.

Electron microscopy

The purified keratan sulphate proteoglycan molecules were fibrillar, as revealed by electron microscopy, exhibiting a high tendency to aggregate. In PBS, samples formed aggregates of up to several microns in length (Fig. 6*a*). In addition, there appeared to be a degree of side-by-side dimerization of molecules in this preparation (Figs. 6*b* and 6*c*). The narrowest width was 3.5 ± 0.5 nm; dimer widths were 7 ± 1 nm.

When samples were prepared in 2 M-guanidine hydrochloride, there was still a high tendency for end-to-end aggregation (Fig. 7*a*), but side-by-side interactions were not observed on any of the grids examined. Again, the narrowest width was 3.0 ± 0.5 nm. However, in some of the grids, much smaller molecules of constant length were observed (Fig. 7*b*). These molecules were 80 ± 10 nm long and were only seen in guanidine hydrochloride preparations. In none of the grids examined, in either preparation, were any glycosaminoglycan filaments observed radiating from the molecules.

Incubation of proteoglycan-coated grids with purified GCTM-2, visualized either by staining with uranyl acetate (Fig. 8) or by rotary shadowing (Fig. 9), clearly showed that the antibody decorates the fibrillar molecules. The samples stained with uranyl acetate are extremely electron-dense and it is difficult to identify individual antibody molecules. However, following rotary shadowing, the GCTM-2 molecules are more clearly defined.

The localization of antibody along the fibrils does not appear to have a regular pattern of distribution.

A control mouse IgM antibody, GCTM-4, did not bind to the fibrils under the same conditions.

DISCUSSION

A keratan sulphate proteoglycan has been isolated from a human embryonal carcinoma cell line by a combination of ion-exchange chromatography and gel filtration. It is an abundant extracellular matrix protein of these cells and of other human embryonal carcinoma cells, such as clonal derivatives of N-Tera 2 (M. F. Pera, unpublished work).

The final preparation was free from contamination with other extracellular matrix proteins known to be produced by these cells [29] as determined by e.l.i.s.a. Incorporation of [^{35}S]sulphate into the purified proteoglycan confirmed that the major sugar components attached to the protein core are sulphated glycan molecules, and not the unsulphated structure, poly-*N*-acetyllactosamine, a series of oligosaccharides that are expressed during specific stages of embryonic development [30].

Both keratanase and chondroitinase treatment of the purified proteoglycan resulted in substantial release of the incorporated [^{35}S]sulphate. After double digestion with these enzymes only 13% of the [^{35}S]sulphate remains attached to the immunoreactive molecule.

The purified preparation ran as a single broad band of molecular mass 200 kDa when subjected to 10% or 7.5% gel electrophoresis, but some immunoreactive material remained in the stacking gel. TFMS hydrolysis completely converts the 200 kDa band into a major band of 55 kDa and a minor 48 kDa band. The 55 kDa band is the major component that remains following deglycosylation. The minor 48 kDa band may be the result of partial hydrolysis during the isolation procedures.

Electron microscopy studies showed a high degree of aggregation of the purified material. A small proportion of the multimers can be dissociated in 2 M-guanidine hydrochloride. Some putative monomers of 80 ± 10 nm in length were observed (Fig. 7*b*). If an extended conformation for the polypeptide chain is assumed, with a molecular mass/length ratio of 330 Da·nm^{-1}, the estimated molecular mass is 26.5 kDa. If however, an α-helical conformation (molecular mass/length ratio of 750 Da·nm^{-1}) is assumed the molecular mass estimation is 60 kDa. This is in close agreement with the estimates for the molecular mass of the core protein from keratanase digestion and TFMS hydrolysis. The width of these images in the electron microscope is 3.0 ± 0.5 nm. Since an α helix plus side-chains is 1.1 nm wide, the images are too thick for this model. While rotary shadowing would have the effect of making the chains appear wider, it may also be that the glycosaminoglycan chains have collapsed onto the sides of the polypeptide core. This was found for certain preparations of the rat chondrosarcoma proteoglycan [31].

The fibrillar molecules described above were clearly identified as the purified antigen by further electron microscopy studies. Purified GCTM-2 is shown bound to the proteoglycan in Figs. 8(*b*) and 8(*c*) and 9(*b*) and 9(*c*). Since GCTM-2 belongs to the IgM immunoglobulin class, each molecule is potentially multivalent. Thus, an individual antibody may interact with epitopes from several different proteoglycan molecules. This may account for the lack of a clear pattern of antibody binding along the fibrils.

The high degree of aggregation revealed by electron microscopy is supported by other biochemical properties of the molecule. Even following double digestion with keratanase and chondroitinase, the material still runs just after the void volume after Superose 6 gel filtration. This suggests that the molecules remain in an aggregated form even after the majority of the glycosaminoglycan chains have been digested away. The mechanism of aggregation is unknown, but since the association is resistant to denaturants, detergents and reducing conditions it may be that a covalent linkage occurs in the cells. The proportion of aggregate versus monomer appears to vary, depending on the confluence of the embryonal carcinoma cells before extraction. Less confluent cells contain a higher proportion of monomer relative to aggregated material.

Amino acid analysis of the proteoglycan revealed a high content of serine, glycine and glutamic acid (plus glutamine). Isolation and partial characterization of rabbit corneal stroma proteoglycans [8] resulted in two keratan-sulphate-rich fractions, PKS-I and PKS-II. Amino acid analysis indicated high contents of aspartic acid, glutamic acid and leucine in PKS-I, whereas PKS-II was rich in aspartic acid, glutamic acid, serine, glycine and lysine. The large aggregating bovine cartilage proteoglycan has a keratan sulphate domain [5]. The amino acid sequence of this region has been determined [6] and consists mainly of a repeating hexapeptide motif which is rich in glutamic acid, serine and proline.

Previous work [15] has shown that keratanase digestion reduces a proportion of the immunoreactive molecules described here to the limiting size of 55 kDa. Whereas the earlier work indicated that the major glycosaminoglycan was keratan sulphate, the results here have clearly shown that chondroitin sulphate is also present. A re-evaluation of the treatment of intact cell monolayers with chondroitinase indicated that this enzyme enhanced the immunoreactivity of the 200 kDa band, but did not reduce the molecular mass significantly. Thus, we conclude that at least a subset of proteoglycan core protein molecules possess keratan sulphate as their major glycosaminoglycan component. For this reason we have termed these antigenic molecules keratan-sulphate-bearing proteoglycans, and have compared their properties with those of previously described keratan sulphate proteoglycans.

Keratanase digestion of bovine corneal proteoglycan [9] resulted in reduction of size from 300 kDa to two bands with apparent molecular masses of 40 kDa and 50 kDa. Recently, this proteoglycan, termed lumican, has been cloned and sequenced [12] and found to code for a protein of 38.6 kDa. Fibromodulin, a keratan sulphate proteoglycan [11], also has a similar-size core protein (42 kDa). Despite the similarities in glycanation and protein core size, the GCTM-2 antigen was not detected in cartilage, skin or cornea [15a]. Moreover, antibodies which react with keratan sulphate proteoglycans from these latter tissues fail to stain human embryonal carcinoma cells (M. F. Pera & S. Cooper, unpublished work). In addition, endo-β-galactosidase from *Escherichia freundii*, an enzyme that degrades keratan sulphate from lumican and fibromodulin, does not cleave keratan sulphate from embryonal keratan sulphate proteoglycan (M. F. Pera & S. Cooper, unpublished work).

Recent work [32,33] suggests that there are several isoforms of the corneal keratan sulphate proteoglycan, of varied core protein size and glycanation. The present study suggests that the keratan sulphate proteoglycan described here also has a varied and complex pattern of glycanation.

The keratan sulphate proteoglycan we have isolated is an abundant molecule on the surface of human embryonal carcinoma cell-lines and is lost upon differentiation *in vitro* [16].

Syndecan, a transmembrane heparan sulphate proteoglycan, is also lost when self-renewing epithelia terminally differentiate [34].

Increasingly, the potential role for proteoglycans in the control of cellular interactions with the extracellular matrix and growth factors is becoming appreciated [3,4]. Many cell attachment

factors such as fibronectin, vitronectin and collagen have glycosaminoglycan-binding domains.

Basic fibroblast growth factor (bFGF) has been shown to associate with the extracellular matrix [35] and basement membrane [36]. Recently, it has been shown that heparan sulphate is necessary for the interaction of bFGF with its receptor [37], indicating a direct role in bFGF cell signalling.

One of the receptors for transforming growth factor β (TGF-β) has been identified as a membrane proteoglycan [38]. Decorin, the small chondroitin sulphate proteoglycan, has been shown to bind TFG-β via its core protein, thereby modulating its activity as a growth factor [39].

It may be that the keratan sulphate embryonal carcinoma proteoglycan described here binds specific attachment factors and/or growth factors important for stem-cell proliferation. Consequently it could be involved in the growth and differentiation of human embryonal carcinoma stem cells. In addition, preliminary studies [17] suggest that a circulating form of the antigen in blood may be a potential stem-cell marker for patients with germ-cell tumours, and is therefore of clinical interest.

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