Isolation of the *N*-terminal globular protein domains from cartilage proteoglycans

Identification of G2 domain and its lack of interaction with hyaluronate and link protein

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The *N*-terminal fragment (G1–G2) of cartilage proteoglycan protein core contains two globular domains, binding region (G1) and a second globular domain (G2). G1–G2 was isolated after mild trypsin digestion of purified proteoglycan aggregates followed by chromatography first on Sepharose CL-2B under associative conditions and then on a TSK-4000 column in 4 M-guanidinium chloride. It migrated as a single band (apparent M_r 150 000) on SDS/polyacrylamide-gel electrophoresis. G2 was isolated by V8-proteinase digestion of G1–G2 followed by aggregation of the G1-containing fragments with hyaluronate and chromatography on TSK-4000. It migrated as a single band on SDS/polyacrylamide-gel electrophoresis of apparent M_r 66 000 after digestion with keratanase. G2 did not interact with proteoglycan monomer, hyaluronate, link protein or other extractable cartilage matrix proteins. A polyclonal antibody raised against G2 did not cross-react with G1 or link protein. These data show that, despite a high degree of sequence similarity, G1 and G2 do not share any functional properties nor have major antigenic sites in common.

INTRODUCTION

The major proteoglycan present in cartilage is a high- M_r aggregating species that contains chondroitin sulphate and keratan sulphate chains, which are located on specific regions of the core protein (Hardingham *et al.*, 1986). The chondroitin sulphate chains (about 100, M_r 15 000–25 000) are restricted to a long extended region of the protein core, whereas the keratan sulphate chains (about 50, M_r 5000–15 000) are distributed more widely throughout the core protein, some on the long chondroitin sulphate-rich region, and on an adjacent keratan sulphate-rich region, but also towards the *N*-terminus around the globular domains G1 and G2 (see below). Some *O*-linked and *N*-linked oligosaccharides are also present.

Three distinct globular domains have been identified in the cartilage proteoglycan core protein by using rotary-shadowing techniques (Wiedemann *et al.*, 1984; Paulsson *et al.*, 1987; Mörgelin *et al.*, 1988), peptide mapping and sequence analysis (Sai *et al.*, 1986; Doege *et al.*, 1986, 1987; Neame *et al.*, 1987; Oldberg *et al.*, 1987) (Fig. 1). These studies revealed two globular domains, G1 and G2 towards the *N*-terminus of the protein core and a *C*-terminal protein domain (G3). The G3 domain (M_r 26 000) contains ten cysteine residues and parts of it have sequence similarity to a hepatic lectin (Doege *et al.*, 1986; Sai *et al.*, 1986; Oldberg *et al.*, 1987; Halberg *et al.*, 1988) and to a β -component found in complement proteins (Patthy, 1987).

The *N*-terminal domain G1 contains a hyaluronate-binding site (Hardingham *et al.*, 1986; Paulsson *et al.*, 1987), and thus represents the region that is involved in specific interactions with hyaluronate and link protein to form stable aggregates of high M_r (Hardingham & Muir,

1972; Heinegård & Hascall, 1974; Hardingham *et al.*, 1976). G1 from pig laryngeal-cartilage proteoglycan contains both *O*-linked and *N*-linked oligosaccharides, some keratan sulphate, but no chondroitin sulphate, and has an apparent M_r of 66 500 (Bonnet *et al.*, 1985). The results of rotary shadowing show that the G2 domain is joined to G1 by a short extended segment (Paulsson *et al.*, 1987) of fixed length (25 nm). G2 shows strong sequence similarity to both link protein and G1 (Neame *et al.*, 1987; Doege *et al.*, 1987). The *N*-terminal regions of G1 and link protein show sequence similarity to an immunoglobulin variable-region fold (Bonnet *et al.*, 1986; Perkins *et al.*, 1989), whereas the more *C*-terminal segments contain a homologous tandem repeat structure. Link protein and G1 thus contain similar structural elements, whereas G2 contains a further copy of the tandem repeat without the immunoglobulin fold. Although link protein and G1 are known to have functions in proteoglycan aggregation, there is no known function for G2.

In the present study we describe the isolation of double-globe (G1–G2) fragment and G2 domain from pig laryngeal-cartilage proteoglycan aggregates, and present evidence that G1 and G2 do not share functional properties.

MATERIALS AND METHODS

Materials

All reagents were analytical grade except for guanidinium chloride (BDH Chemicals, Poole, Dorset, U.K.), which was purified with activated charcoal (Norit GSX; Hopkin and Williams, Chadwell Heath, Essex, U.K.).

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Fig. 1. Schematic representation of proteoglycan core protein structure

The globular hyaluronate-binding region (G1) is joined to a second globular domain, G2, by a short extended region (E1). The region designated E2 represents the long extended region of the core protein that bears most of the keratan sulphate (KS) chains including the keratan sulphate-rich region and all of the chondroitin sulphate (CS) chains. G3 represents the C-terminal globular domain. The Ig fold (A loop) and the two copies of the tandem repeat structures (B and B' loops) present in the G1 and G2 sequence are shown schematically.

Trypsin (diphenylcarbamoyl chloride-treated) (EC 3.4.21.4), 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, disodium EDTA, benzamidine hydrochloride, bovine serum albumin (fraction V), V8 proteinase (*Staphylococcus aureus* strain 8), soya-bean trypsin inhibitor, 3,3'-diaminobenzidine tetrahydrochloride, anti-(mouse IgG) antibody-peroxidase and anti-(rabbit IgG) antibody-peroxidase conjugates, *Staphylococcus aureus* (Cowan strain) powdered cells and iodoacetamide were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Sepharose CL-2B, DEAE-Sephacel and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). TSK-3000 and TSK-4000 h.p.l.c. columns were obtained from Anachem (Luton, Beds., U.K.); Endo- β -D-galactosidase (keratanase) was obtained from Miles Laboratories (Slough, Berks., U.K.). Casein was obtained from Fisons Scientific Apparatus (Loughborough, Leics., U.K.) and skim-milk powder (Marvel) was commercially available. Na¹²⁵I (IMS 30) was obtained from Amersham International (Amersham, Bucks., U.K.). Hyaluronate-Sepharose was a gift from Michael Holmes and Michael Bayliss of this Institute.

Preparation of cartilage proteoglycan and collagen

Proteoglycan aggregates extracted from pig laryngeal cartilage in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors were purified in a CsCl density gradient as described by Hardingham (1979). The gradients were cut into three equal fractions. Proteoglycans were isolated from the bottom fraction (A1), and fractions A2 and A3 containing other cartilage components were kept for interaction experiments (see below). Proteoglycan monomer (A1D1) and link protein were prepared from proteoglycan aggregate by fractionation in a dissociative CsCl density gradient (Hardingham, 1979) and link protein was further purified by chromatography on a TSK-3000 (30 cm $\times$ 2.1 cm) column in 4 M-guanidinium chloride. Type II collagen from pig laryngeal cartilage was prepared by solubilization with pepsin (Miller, 1972) and salt precipitation as described by Grant *et al.* (1988).

Preparation of double-globe domain (G1-G2)

Purified freeze-dried proteoglycan aggregate (A1) was dissolved overnight in 0.1 M-Tris/acetate buffer, pH 7.3 (15 mg/ml), then digested with trypsin for 2 h at 37 °C at a final concentration of 3 μ g of trypsin/150 mg of A1. The reaction was stopped by the addition of a 100-fold excess of trypsin inhibitor and concentrated by partial freeze-drying to one-third volume for chromatography on a Sepharose CL-2B column (2 cm $\times$ 140 cm) in 0.5 M-sodium acetate buffer, pH 6.8, at 4 °C. Fractions were assayed for binding region (G1), link protein and keratan sulphate by radioimmunoassay (see below), and for sulphated glycosaminoglycans by the dye-binding assay (Ratcliffe *et al.*, 1988). The void-volume (V_0) fractions were pooled, dialysed against distilled water and freeze-dried. The sample was then dissolved in 4 M-guanidinium chloride/1 mM-sodium EDTA/50 mM-sodium acetate buffer, pH 5.8, and chromatographed on a TSK-4000 column (0.75 cm $\times$ 60 cm) eluted in the same buffer to separate the G1-G2 fragment from G1 and link protein. Fractions were analysed for link protein, G1 and keratan sulphate by radioimmunoassay (see below), and the pooled fractions containing G1-G2 were rechromatographed on the same column, dialysed and freeze-dried.

Preparation of globular domain G2

Purified G1-G2 fragment (520 μ g) was dissolved in 10 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.0, and digested for 1 h at 37 °C with 0.452 unit of V8 proteinase in a total volume of 416 μ l. The digestion was stopped by boiling for 5 min and 10 μ l of hyaluronate (10 mg/ml) was added, and the mixture was kept overnight at 4 °C. Half the sample was applied to a TSK-4000 column (30 cm $\times$ 0.75 cm) eluted in phosphate-buffered saline (0.123 M-NaCl/10 mM-Na₂HPO₄/3.16 mM-KH₂PO₄ buffer, pH 7.4), and monitored for absorbance at 280 nm. The fractions were assayed for G1 and keratan sulphate epitopes (see below). A u.v.-absorbing peak (containing G2) that was included on the column and that did not contain any G1 epitope was pooled and purified by rechromatography on the same column in the presence of extra hyaluronate (100 μ g). The included

G2 peak was pooled, dialysed and freeze-dried. In some instances G1–G2 was digested with keratanase (0.07 unit/mg in 50 mM-Tris/HCl/50 mM-sodium acetate buffer, pH 7.4, for 3 h at 37 °C) before V8-proteinase digestion.

With some G2 preparations an additional ion-exchange purification step was included. G2 samples (about 0.5 mg) were dissolved in 200 μ l of 0.2 M-NaCl/50 mM-sodium acetate buffer, pH 6.8, and applied to a DEAE-Sephacel column (0.9 cm $\times$ 1.4 cm). The sample was eluted with 5 ml each of 0.2 M-, 0.4 M-, 0.6 M-, 0.8 M-, 1.0 M-, 1.5 M- and 2.0 M-NaCl, all in 50 mM-sodium acetate buffer, pH 6.8. Each 5 ml fraction was assayed for G2 and keratan sulphate epitope, and the purified G2 (see below) fraction was dialysed and freeze-dried.

Immunoprecipitation of V8-proteinase-digestion products

Portions (10⁵ c.p.m./10 μ l) of ¹²⁵I-labelled control or V8-proteinase-digested G1–G2 samples were mixed with 15 μ l of anti-G1 serum (the amount shown to produce maximum precipitation on a titration curve) and incubated overnight at 4 °C in incubation buffer composed of phosphate-buffered saline, pH 7.4, containing 12 mM-deoxycholate, 0.1 % Nonidet, 0.1 % bovine serum albumin and 0.03 % NaN₃. Antibody–antigen complexes and free antibody were precipitated by the addition of 200 μ l of 10 % (w/v) *Staphylococcus aureus* dried cells, and were separated from the supernatants by centrifugation. Each precipitate was extracted twice by boiling for 5 min in 50 μ l of 10 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8, and 1 μ l of 1 % (w/v) SDS and the extracts were combined.

Iodination and radioimmunoassays

Iodination of G1, G2, G1–G2, link protein and proteoglycan monomer was performed as described by Greenwood *et al.* (1963) with chloramine-T as the oxidizing agent. Approx. 5 μ g of protein was iodinated with 0.3 mCi of Na¹²⁵I, and the iodinated product was stored at –20 °C. Radioimmunoassays for G1 and link protein were performed with polyclonal antisera and for keratan sulphate with the monoclonal antibody MZ-15 as described previously (Ratcliffe & Hardingham, 1983; Zanetti *et al.*, 1985). A competitive radioimmunoassay for G2 was developed with the polyclonal antiserum raised in the present study. The assay method was similar to that previously described (Ratcliffe & Hardingham, 1983) but with ¹²⁵I-labelled G1–G2 as antigen.

Determination of sulphated glycosaminoglycans

The concentration of sulphated glycosaminoglycans in Sepharose CL-2B fractions was determined by a modification of the dye-binding assay (Farndale *et al.*, 1982) carried out in 96-well micro-titre plates (Ratcliffe *et al.*, 1988). Portions (40 μ l) of samples or standards in 0.5 M-sodium acetate buffer, pH 6.8, were placed in wells and the plates were read at 600 nm on a Bio-Rad model 2550 EIA reader immediately after the addition of 250 μ l of dye solution.

SDS/polyacrylamide-gel electrophoresis, immunoblotting and autoradiography

Electrophoresis was performed in 5 % polyacrylamide gels as described by Fairbanks *et al.* (1971). In some instances samples were reduced by boiling in the presence of 2 μ l of 2-mercaptoethanol before being loaded on to

the gels. Protein bands were detected by silver staining (Morrissey, 1981).

Immunoblotting of protein antigens was performed by a modification of the method of DeBlas & Cherwinski (1983). Proteins were electrophoretically transferred from polyacrylamide slab gels on to nitrocellulose sheets (Schleicher und Schüll, Dassel, West Germany) overnight at 80 mA at 4 °C in transfer buffer (2 mM-EDTA/40 mM-Tris/HCl/20 mM-sodium acetate buffer, pH 7.4). The nitrocellulose sheets were blocked with either 2 % (w/v) casein or 5 % (w/v) skim-milk powder in phosphate-buffered saline. Antibody dilutions were 1:1000 for anti-G1 antibody, 1:500 for anti-G2 antibody and 1:500 for anti-(link protein) antibody. After six washes (2–5 min) in phosphate-buffered saline, sheets were incubated in 1:1000 dilution of anti-(mouse IgG) antibody–peroxidase or anti-(rabbit IgG) antibody–peroxidase conjugate for 1 h at room temperature. The peroxidase reaction was developed in the dark as described by DeBlas & Cherwinski (1983) with 3,3'-diaminobenzidine tetrahydrochloride as substrate.

Slab gels containing ¹²⁵I-labelled material were vacuum-dried and exposed for 16–48 h at –70 °C to X-Omat S X-ray film (Kodak, Hemel Hempstead, Herts., U.K.). Developed X-ray films were scanned on a Shimadzu CS-930 dual-wavelength scanner.

Preparation of anti-G2 serum

Antisera were raised in rabbits (Ratcliffe & Hardingham, 1983) by primary injection of 160 μ g of G2 in Freund's complete adjuvant followed by booster injections of 30–80 μ g of G2 in Freund's incomplete adjuvant at approx. 3-week intervals. Keratanase-digested G2 was used for some later boosts. Antisera were collected 10–12 days after each boost.

G1–G2 and G2 interaction experiment

The proportion of ¹²⁵I-labelled G1–G2 that had retained its ability to bind to hyaluronate after iodination was selected by passing a portion (4 $\times$ 10⁶ c.p.m.) over a 200 μ l hyaluronate–Sepharose column in 1 M-NaCl/50 mM-sodium acetate buffer, pH 6.8, and eluting the fraction (38 %) that bound to the gel with 4 M-guanidinium chloride in the same buffer. ¹²⁵I-labelled G1–G2 or ¹²⁵I-labelled G2 (keratanase-digested) was then mixed with either 125 μ g of proteoglycan monomer with or without 10 μ g of hyaluronate with or without 5 μ g of link protein, or 100 μ g of A2 fraction, or 100 μ g of A3 fraction, or 100 μ g of type II collagen, in a total volume of 400 μ l. Each sample was made up to a final concentration of 4 M-guanidinium chloride in order to dissociate all components and then dialysed against several changes of phosphate-buffered saline to permit reassociation. For testing interaction with type II collagen samples were mixed directly with it in phosphate-buffered saline. Each sample was chromatographed on an associative TSK-4000 column, and the fractions were assayed for radioactivity and total glycosaminoglycan content where appropriate.

Identical experiments were performed with 10 μ g of non-iodinated G2 that had been digested with keratanase and then mixed with either 250 μ g of proteoglycan monomer with or without 40 μ g of hyaluronate with or without 10 μ g of link protein, or 200 μ g of A2 fraction, or 200 μ g of A3 fraction, or 200 μ g of type II collagen, in a total volume of 200 μ l. These samples were eluted from

a Sepharose CL-2B column (12.5 cm $\times$ 0.8 cm) in 0.123 M-NaCl/50 mM-sodium acetate buffer, pH 7.4, and the fractions were assayed for G2 epitope.

RESULTS

Previous results have shown that trypsin cleaves the proteoglycan protein core (Fig. 1) at many sites, but that the globular binding-region domain and link protein are resistant to digestion if they are part of a stable aggregate

structure bound to hyaluronate (Heinegård & Hascall, 1974; Bonnet *et al.*, 1985). In preliminary experiments less extensive digestion was shown to release a core-protein fragment containing two globular domains, which were identified by rotary-shadowing electron microscopy (Paulsson *et al.*, 1987). In the present study this double-globe fragment was prepared by mild trypsin digestion of pig laryngeal-cartilage proteoglycan aggregates. Fractionation of the digest on Sepharose CL-2B separated all the proteoglycan fragments remaining

Fig. 2. Sepharose CL-2B chromatography of trypsin-digested proteoglycan aggregate

Proteoglycan aggregate was digested with trypsin as described in the Materials and methods section, and the digest was chromatographed on Sepharose CL-2B in 0.5 M-sodium acetate buffer, pH 6.8, at 4 °C. Fractions were assayed for sulphated glycosaminoglycans by the dye-binding assay (○) and for G1 (●), link protein (▲) and keratan sulphate (■) by radioimmunoassays. The keratan sulphate epitope was determined by competition with a purified proteoglycan monomer from pig laryngeal cartilage and results are expressed in mol of proteoglycan (Zanetti *et al.*, 1985). The V_0 fractions were pooled for chromatography on a dissociative TSK-4000 column (Fig. 3).

Fig. 3. TSK-4000 h.p.l.c. of hyaluronate-bound trypsin-digested proteoglycan aggregate after mild trypsin digestion

Hyaluronate-bound fractions recovered in the V_0 fraction of a Sepharose CL-2B chromatography (Fig. 2) after mild trypsin digestion were dissociated in 4 M-guanidinium chloride and fractionated on a TSK-4000 h.p.l.c. column as described in the Materials and methods section. The A_{280} (---) was measured across the profile and portions of each fraction were diluted 1:100 and assayed for G1 (●), link protein (△) and keratan sulphate (■) (see Fig. 2) by radioimmunoassay.

bound to hyaluronate in the V_0 fraction of the column from the large glycosaminoglycan-rich fragments cleaved from the proteoglycans. Radioimmunoassays showed that the bound fraction contained all the binding region (G1) and link protein together with 30% of the total keratan sulphate epitope, but only 4–10% of the total glycosaminoglycans (Fig. 2).

The hyaluronate-bound fraction was dissociated in 4 M-guanidinium chloride and fractionated by size exclusion on a TSK-4000 h.p.l.c. column (Fig. 3). Five main protein peaks were detected, which were identified by radioimmunoassays for G1, keratan sulphate and link protein. In addition to a fraction of similar size to trypsin-prepared binding region (G1) peak IV, there was also a peak (III) of much higher M_r that contained G1, which was shown by rotary-shadowing electron microscopy to contain a double-globe structure (Paulsson *et al.*, 1987). After rechromatography on the same column

this fraction migrated as a single broad band on SDS/5%-polyacrylamide-gel electrophoresis with an apparent M_r of 150 000 (Fig. 4). Although less than 50% of the total G1 was in this fraction, shorter digestion times did not increase the yield (results not shown). A larger fragment containing G1 was also present (peak II), which may correspond to G1–G2 with an additional protein core segment attached to it, but this has not been characterized further.

The iodinated G1–G2 fragment was immunoprecipitated by polyclonal anti-G1 serum and also by a monoclonal anti-(keratan sulphate) antibody, showing that the G1 epitopes survived iodination and that keratan sulphate was attached to the double-globe structure.

V8-proteinase digestion of G1–G2

The structure of the G1–G2 fragment was investigated by using V8 proteinase, which hydrolyses peptide and

Fig. 4. SDS/polyacrylamide-gel electrophoresis of V8-proteinase-digested G1–G2

^{125}I -labelled G1–G2 (100 μl) was digested at 37 °C for 1 h in 10 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.0, with 1.45×10^{-3} , 1.45×10^{-2} or 1.45×10^{-1} unit of V8 proteinase/200 μl digestion volume (a–c). $\cdots\cdots$, Undigested G1–G2 (corresponding to fragment structure 0 in Fig. 5); $-\cdots-$, digest products. After being boiled for 2 min, portions of each digest were immunoprecipitated with anti-G1 serum, and the ^{125}I -labelled fragments present in the supernatants and pellets were separated as described in the text (d–f). Portions of each digest and the corresponding supernatants (----, G1-negative) and precipitates (—, G1-positive) were subjected to SDS/5%-polyacrylamide-gel electrophoresis under non-reducing conditions. The profiles were obtained by scanning autoradiographs of the dried gels. Arrows at the top of each frame indicate M_r markers corresponding to M_r 150 000, 60 000 and 20 000 respectively.

Fig. 5. Schematic representation of the G1-G2 fragment structures produced by V8-proteinase digestion

The proposed composition and approximate M_r of the fragments are shown. The numbers of fragments (0 to 7) correspond to the peak numbers given in Fig. 4. G1-positive fragments are those that contain G1, and G1-negative fragments are those that do not.

ester bonds specifically at the carboxy end of glutamic acid and aspartic acid residues (Drapeau *et al.*, 1972). ^{125}I -labelled G1-G2 was digested with three concentrations of V8 proteinase (Figs. 4a-4c), and portions of each digest were immunoprecipitated with anti-G1 serum in order to separate fragments containing G1 epitopes (G1-positive) from those that did not (G1-negative). The total digests were run on SDS/polyacrylamide-gel electrophoresis (Figs. 4a-4c) and were compared with the immunoprecipitated (G1-positive) fragments and those that remained in the supernatant that were run separately (Figs. 4d-4f). The total digests showed a progressive decrease in the size of the fragments produced with increasing concentrations of V8 proteinase. At the lowest concentration, immunoprecipitation with anti-G1 serum identified undegraded G1-G2 (structure 0, M_r 150000; Fig. 5) and a major fragment of M_r approx. 60000 (Fig. 4d, peak 4). At a 10-fold greater concentration of V8 proteinase (Figs. 4b and 4e) the G1 fragments were present almost entirely as 75000- M_r and 34000- M_r products (Fig. 4e, peaks 3 and 6 respectively), and at a 100-fold greater concentration of enzyme (Figs. 4c and 4f) G1 was present as 34000- M_r and smaller products (Fig. 4f, peaks 6 and 7 respectively).

At the lowest concentration of V8 proteinase, most of the binding-region-negative (G1-negative) material, i.e. that which was not immunoprecipitated by anti-G1 serum, was present as a large fragment (M_r 110000), which corresponded to the G1-G2 fragment less the G1 domain. With increasing amounts of V8 proteinase the 110000- M_r fragment was converted into a 45000- M_r fragment (Figs. 4e and 4f). Fig. 5 shows schematically the proposed composition of the fragments generated by V8-proteinase digestion of G1-G2 as deduced from the results shown in Figs. 4(d)-(4f).

Isolation of G2 from the G1-G2 fragment and preparation of anti-G2 serum

The G1-G2 fragment was digested with V8 proteinase, and excess hyaluronate was added. Size-exclusion

Fig. 6. Isolation of G2 on TSK-4000

G2 was cleaved from purified G1-G2 fragment by V8 proteinase, and purified from G1 by h.p.l.c. on TSK-4000 in phosphate-buffered saline, pH 7.4, after allowing any fragments containing G1 to bind to added hyaluronate. The column was monitored for A_{280} (Δ) and the fractions were assayed for G1 ($\bullet$) and keratan sulphate ($\blacktriangle$) by radioimmunoassay.

Fig. 7. SDS/polyacrylamide-gel electrophoresis of purified G2

Purified G2, which had been prepared by digesting 1.18 mg of keratanase-digested G1-G2 with 0.858 unit of V8 proteinase (final volume 800 μl) and isolated on an associative TSK-4000 column as described in the text, was subjected to SDS/7.5% polyacrylamide-gel electrophoresis in the presence (track 2) or in the absence (track 1) of 2-mercaptoethanol. Protein bands were detected by silver staining.

chromatography on a TSK-4000 column separated components containing G1 that were bound to hyaluronate, which were excluded from the column, from G2, which was included in the column (Fig. 6). The elution profile of keratan sulphate epitope on the TSK 4000 column suggested that the G2 fragments released were a hetero-

Fig. 8. Specificity of rabbit polyclonal antiserum to G2

A rabbit polyclonal anti-G2 serum was tested for specificity by comparing competitive binding of A1D1 (▲), G1-G2 (■), link protein (□) and G1 (△) in a radioimmunoassay. Dilutions of each competitor (100 μ l) were incubated overnight at room temperature with 1:50 dilution of 125 I-G1-G2 (keratanase-digested) and 1:80 dilution of anti-serum.

geneous population with some containing much more keratan sulphate epitope than others. After keratanase digestion, which removes about 90% of the keratan sulphate epitope, G2 migrated as a single band on SDS/7.5%-polyacrylamide-gel electrophoresis with an apparent M_r of 66000 for the native molecule and M_r 70000 under reducing conditions (Fig. 7). The slight increase in apparent size on reduction showed the G2 fragment to contain a single polypeptide without internal cleavages.

The 66000- M_r fragment isolated was thus identified as containing G2, as it was derived as a large fragment of G1-G2 and did not contain any antigenic determinants found on G1. The increase in mobility on electrophoresis showed it to be at least partially globular. Further experiments have shown it to react with a monoclonal antibody 1-C-6 (Hejna *et al.*, 1987), which has been reported to recognize a peptide sequence found in G1 and repeated in G2 in the rat. Reaction of antibody 1-C-6 with G2 only occurred after reduction and keratanase digestion (A. J. Fosang & T. E. Hardingham, unpublished work). The 66000- M_r fragment thus contained a buried epitope recognizable by antibody 1-C-6 but none of the major antigenic determinants found on G1, and it must therefore contain at least a major part of the G2 domain.

A rabbit polyclonal anti-G2 serum was raised against purified pig G2. It showed specificity for G2 and did not cross-react with other proteoglycan components after electrophoresis and immunoblotting (results not shown). Link protein and G1 showed only low levels of competition at high concentrations (30 μ g/ml) in a radioimmunoassay of G2 (Fig. 8). The major epitopes on G2 were therefore absent from G1 and link protein. The antiserum appeared to be species-specific and did not show significant cross-reactivity with dog or human proteoglycan monomer, before or after keratanase digestion (results not shown).

Fig. 9. Purification of G2 by ion-exchange chromatography

G2 was prepared as described in the Materials and methods section (a) or prepared from keratanase-digested G1-G2 (b) as shown in Fig. 7 and applied to a DEAE-Sephacel column. The sample was eluted in a stepwise manner in 50 mM-sodium acetate buffer, pH 6.8, containing 0.2 M- to 2.0 M-NaCl and assayed for G2 epitope (□) and keratan sulphate epitope (▨). The residual keratan sulphate epitope present on keratanase-treated G2 represents 10% or less of the total keratan sulphate that was present on G1-G2 before keratanase digestion.

Ion-exchange fractionation of G2

Keratanase-digested G2 was fractionated by ion-exchange chromatography and the fractions were monitored for G2 by using a radioimmunoassay (see below) and for residual keratan sulphate epitopes (Fig. 9b). The fraction that was eluted from DEAE-Sephacel with 0.4 M-NaCl contained 60% of the G2 epitopes and only 7% of the total keratan sulphate epitope, whereas fractions eluted at salt concentrations at and above 0.8 M contained 86% of the keratan sulphate epitope and only 15% of G2. The results show that a small proportion of G2 (< 10%) contains some keratan sulphate epitope that is resistant to keratanase digestion. In comparison, the elution of G2 untreated with keratanase was in the region of 0.6 M- and 0.8 M-NaCl (Fig. 9a). This indicated that all G2 fragments contained some keratan sulphate, even though the 0.6 M-NaCl fraction contained no detectable keratan sulphate epitopes. The monoclonal antibody has been shown to recognize an oversulphated keratan sulphate sequence (Mehmet *et al.*, 1986), and this may be absent from these keratan sulphate chains.

Table 1. Interaction of ^{125}I -labelled G1-G2 and ^{125}I -labelled G2 with proteoglycan and cartilage components

^{125}I -labelled G1-G2 or ^{125}I -labelled G2 was allowed to interact with either proteoglycan monomer with or without hyaluronate with or without link protein, or A2 fraction, or A3 fraction (density-gradient fractions containing cartilage matrix proteins), or type II collagen, and eluted on an associative TSK-4000 column as described in the Materials and methods section. The binding was determined from the shift in the radioactivity of the labelled component to the V_0 of the column.

Components	Binding of ^{125}I -G1-G2 (%)	Binding of ^{125}I -G2 (%)
Proteoglycan monomer	22	1
Proteoglycan + hyaluronate	76	2
Proteoglycan + hyaluronate + link protein	88	2
A3 fraction	72	1
A2 fraction	41	1
Type II collagen	0	0

Interaction of N-terminal globular domains with extracted cartilage components

Link protein and G1 are known to have a role in aggregate formation by their non-covalent binding to both hyaluronate and each other. Since G2 also contained a sequence similarity (Neame *et al.*, 1987; Doege *et al.*, 1987) to the tandem repeat found in G1 and link protein, it was expected that G2 might share some of their functional properties. The ability of ^{125}I -labelled G1-G2 fragment and ^{125}I -labelled G2 to interact with hyaluronate in the presence of link protein and proteoglycan monomer was investigated by mixing the components under associative conditions and chromatographing on a TSK-4000 column. Similar experiments were performed with type II collagen and a mixture of 4 M-guanidinium chloride-extractable cartilage matrix components from which most of the proteoglycan had been removed. ^{125}I -labelled G1-G2 showed a low degree of interaction with proteoglycan (22%), but a high proportion bound with proteoglycan and hyaluronate (76%), and even more with proteoglycan, hyaluronate and link protein (88%). These results showed the ability of G1-G2 to participate in the interactions of aggregation. In contrast, ^{125}I -labelled G2 showed negligible interaction (1-2%) with any of these components, indicating that G2 has no affinity for link protein, proteoglycan (or G1) or hyaluronate. It thus had no properties comparable with those of isolated G1 (Bonnet *et al.*, 1985) or the G1-G2 fragment. ^{125}I -labelled G1-G2 also showed interaction with mixed matrix protein fractions A2 (41%) and A3 (72%), as these contained variable proportions of free link protein and free hyaluronate as well as other matrix macromolecules. The labelled G2 fragment showed no interaction with A2 or A3 fractions, and it therefore had no detectable binding with other extracted matrix proteins.

Identical interaction experiments were also performed with non-radiolabelled keratanase-digested G2 as described in the Materials and methods section. In each case there was no interaction of G2 with any of the

components tested, indicating that the iodinated protein showed properties that were similar to those of the non-iodinated protein. The binding of G1-G2 to link protein and hyaluronate therefore appears to be mediated exclusively through the G1 domain (Table 1). Neither G1-G2 nor G2 showed detectable binding to pepsin-digested type II collagen.

DISCUSSION

The sequence data show that considerable similarity exists between the primary and secondary structures of G1, G2 and link protein (Neame *et al.*, 1987; Doege *et al.*, 1987; Perkins *et al.*, 1989). A double-loop structure for each of G1, G2 and link protein has been proposed, as well as an Ig fold, which is present in G1 and link protein. Both G1 and link protein interact specifically with a decasaccharide segment of hyaluronate and also bind to each other (Hardingham & Muir, 1973; Hascall & Heinegård, 1974; Bonnet *et al.*, 1985; Périn *et al.*, 1987). The present results show that in spite of containing a double-loop structure the G2 domain does not have any binding properties similar to those of G1 or link protein. Furthermore, it also shows no evidence of binding with type II collagen or guanidinium chloride-solubilized cartilage matrix proteins. The lack of interaction of G2 with link protein or hyaluronate is entirely compatible with rotary-shadowing electron-microscopic studies (Paulsson *et al.*, 1987; Mörgelin *et al.*, 1988) on the G1-G2 fragment that suggested that only one globular domain G1 was involved in binding to hyaluronate and link protein. The non-binding globular domain is thus identified as G2.

Although the G2 domain contains similar sequences to those found in G1 and link protein, the polyclonal antiserum raised against it did not show cross-reaction with G1 or link protein. It has previously been shown that polyclonal anti-G1 and anti-(link protein) sera also showed no cross-reaction between their respective antigens (Ratcliffe & Hardingham, 1983). Thus, in spite of containing similar sequences, they are certainly not identical and the differences in sequence must be sufficient to ensure that the major antigenic determinants are unique to each protein structure and not shared with the related proteins.

The results reveal no function for G2 or offer no reason for the presence of a partial repeat of G1 in a separate G2 domain. It may act as a structural element that helps separate the major glycosaminoglycan-bearing region from the site of aggregation and thereby make it more favourable to aggregation. It may present a site for proteolysis involved in turnover of the extracellular matrix. Studies of the fragments produced in turnover have shown cleavage close to G2 to be a common early site of attack (Ratcliffe *et al.*, 1986). As the G1 domain has been shown to be fully competent for stable interaction with hyaluronate and link protein and the present results detect no comparable activities in G2, it would appear that the G2 domain is not involved in proteoglycan aggregation or interaction with other cartilage matrix proteins.

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REFERENCES

- Bonnet, F., Dunham, D. G. & Hardingham, T. E. (1985) *Biochem. J.* **228**, 77–85
- Bonnet, F., Périn, J.-P., Lorenzo, F., Jollès, J. & Jollès, P. (1986) *Biochim. Biophys. Acta* **873**, 152–155
- DeBlas, A. L. & Cherwinski, H. M. (1983) *Anal. Biochem.* **133**, 214–219
- Doege, K., Fernandez, P., Hassell, J. R., Sasaki, M. & Yamada, Y. (1986) *J. Biol. Chem.* **261**, 8108–8111
- Doege, K., Sasaki, M., Horigan, E., Hassell, J. R. & Yamada, Y. (1987) *J. Biol. Chem.* **262**, 17757–17767
- Drapeau, G. R., Boily, Y. & Houmard, J. (1972) *J. Biol. Chem.* **247**, 6720–6726
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2616
- Farndale, R. W., Sayers, C. A. & Barrett, A. J. (1982) *Connect. Tissue Res.* **9**, 247–248
- Grant, M. E., Ayad, S., Kwan, A. P. L., Bates, G. P., Thomas, J. T. & McClure, J. (1988) in *The Control of Tissue Damage* (Glauert, A. M., ed.), pp. 3–28, Elsevier, Amsterdam, New York and Oxford
- Greenwood, E. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Halberg, D. F., Proulx, G., Doege, K., Yamada, Y. & Drickamer, K. (1988) *J. Biol. Chem.* **263**, 9486–9490
- Hardingham, T. E. (1979) *Biochem. J.* **177**, 237–247
- Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
- Hardingham, T. E. & Muir, H. (1973) *Biochem. J.* **135**, 905–908
- Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127–143
- Hardingham, T. E., Beardmore-Gray, M., Dunham, D. G. & Ratcliffe, A. (1986) *Ciba Found. Symp.* **124**, 30–46
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4241–4249
- Heinegård, D. & Hascall, V. C. (1974) *J. Biol. Chem.* **249**, 4250–4256
- Hejna, M., Kimura, J., Caterson, B., Hascall, V., Stevens, J., Oike, Y., Tripiet, D. & Handley, C. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 1961
- Mehmet, H., Scudder, P., Tang, P. W., Hounsell, E. F., Caterson, B. & Feizi, T. (1986) *Eur. J. Biochem.* **157**, 385–391
- Miller, E. J. (1972) *Biochemistry* **11**, 4903–4909
- Mörgelin, M., Paulsson, M., Hardingham, T. E., Heinegård, D. & Engel, J. (1988) *Biochem. J.* **253**, 175–185
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Neame, P. J., Christner, J. E. & Baker, J. R. (1987) *J. Biol. Chem.* **262**, 17768–17778
- Oldberg, A., Antonsson, P. & Heinegård, D. (1987) *Biochem. J.* **243**, 255–259
- Patthy, L. (1987) *J. Mol. Biol.* **198**, 567–577
- Paulsson, M., Mörgelin, M., Wiedemann, H., Beardmore-Gray, M., Dunham, D., Hardingham, T., Heinegård, D., Timpl, R. & Engel, J. (1987) *Biochem. J.* **245**, 763–772
- Périn, J.-P., Bonnet, F., Thurieu, C. & Jollès, P. (1987) *J. Biol. Chem.* **262**, 13269–13272
- Perkins, S. J., Nealis, A., Dudhia, J. & Hardingham, T. E. (1989) *J. Mol. Biol.* **206**, 737–754
- Ratcliffe, A. & Hardingham, T. (1983) *Biochem. J.* **213**, 371–378
- Ratcliffe, A., Tyler, J. A. & Hardingham, T. E. (1986) *Biochem. J.* **238**, 571–580
- Ratcliffe, A., Doherty, M., Maini, R. N. & Hardingham, T. E. (1988) *Ann. Rheum. Dis.* **47**, 826–832
- Sai, S., Tanaka, T., Kosher, R. A. & Tanzer, M. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5081–5085
- Wiedemann, H., Paulsson, M., Timpl, R., Engel, J. & Heinegård, D. (1984) *Biochem. J.* **224**, 331–333
- Zanetti, M., Ratcliffe, A. & Watt, F. M. (1985) *J. Cell Biol.* **101**, 53–59

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