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Structure and interactions of cartilage proteoglycan binding region and link protein

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Binding region and link protein were prepared from pig laryngeal cartilage proteoglycans after chondroitinase ABC and trypsin digestion. Experiments on gel chromatography showed the purified binding region to interact reversibly with hyaluronate (HA), and this binding was also shown to be stabilized by native link protein. The trypsin-prepared link protein showed properties of self-association in solution that were partially inhibited by oligosaccharides (HA_{10-16}) and abolished by modification of free amino groups (lysine residues) with 2-methylmaleic anhydride. The M_r (sedimentation equilibrium) of the modified link protein was 41700. Analysis of binding region showed it to contain 25% (w/w) carbohydrate, mainly in galactose, glucosamine, mannose and galactosamine. It contained some keratan sulphate, as digestion with endo-β-D-galactosidase (keratanase) removed 28% galactose and 25% glucosamine and the M_r (sedimentation equilibrium) decreased from 66500 to 60800. After keratanase digestion the interaction with polyclonal antibodies specific for binding region was unaffected, but the response in a radioimmunoassay with a monoclonal antibody to keratan sulphate was decreased by 47%. Preparation of a complex between binding region, link protein and $HA_{\sim 34}$ showed a single component (5.5S) of M_r (sedimentation equilibrium) 133500. In this complex the antigenic determinants of link protein appeared masked, as previously found with proteoglycan aggregates. The isolated binding region and link protein were thus shown to retain properties comparable with those involved in the structure and organization of proteoglycan aggregates.

The major proteoglycans of cartilage are of high M_r and aggregate by binding to hyaluronate (Hardingham & Muir, 1972) via a site on a globular domain (Heinegård & Hascall, 1974; Hardingham *et al.*, 1976) at the *N*-terminus of the proteoglycan protein core. The native aggregate contains in addition link protein, one molecule of which is bound stoichiometrically to each proteoglycan and also interacts with hyaluronate (Heinegård & Hascall, 1974). This greatly increases the stability of the aggregate and effectively locks each proteoglycan on to the hyaluronate chain (Hardingham, 1979; Tang *et al.*, 1979). The binding region of proteoglycan interacts strongly with a decasaccharide or larger unit of hyaluronate (K_D 2 × 10^{-8} M) (Christner *et al.*, 1978; Cleland, 1979; Nieduszynski *et al.*, 1980) and is highly specific, as it shows no interaction with chondroitin (Hascall & Heinegård, 1974) or other polyanions (Hardingham & Muir, 1972). Link protein binds to a decasaccharide unit of hyaluronate (Tengblad, 1981) and occurs in two molecular-mass forms, where the larger ($M_{r(app.)}$ 48000) contains additional carbohydrate not present on the smaller form ($M_{r(app.)}$ 43000) (Bonnet *et al.*, 1978; Baker & Caterson, 1979; Le Glédic *et al.*, 1983).

Digestion of the native aggregate with trypsin cleaves the proteoglycan, but leaves intact two large protein fragments (Heinegård & Hascall, 1974), one of which is derived from the hyaluronate-binding region of proteoglycan and the other corresponds to a major-link protein fragment just smaller than the native small form. The abundance of the two native forms of link protein varies among different species (Baker & Caterson, 1979) and pig laryngeal cartilage contains mainly

Abbreviation used: SDS, sodium dodecyl sulphate.

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(approx. 90%) the larger form (Hardingham *et al.*, 1981).

Although the binding region of proteoglycan represents a domain of low carbohydrate content compared with the other regions of the protein core (Heinegård & Hascall, 1974), analysis of that isolated from pig laryngeal proteoglycan showed the carbohydrate content to be 30% (w/w) and suggested it to contain both *O*-linked and *N*-linked oligosaccharides and some keratan sulphate, although no chondroitin sulphate was present (Perkins *et al.*, 1981). Investigation of its low-angle neutron scattering in solution yielded a low-resolution model, which suggested an elongated prolate ellipsoid (semi-axes 7.5nm × 1.5nm × 1.5nm) (Perkins *et al.*, 1981). Estimates of the M_r from these results were imprecise and, in view of the high carbohydrate content, those from SDS/polyacrylamide-gel electrophoresis were also unlikely to be reliable. The present work was thus initiated to investigate further the structure and interactions of binding region and link protein and to determine their M_r values.

Materials and methods

Materials

All reagents were of analytical grade, except for carbazole and guanidinium chloride (BDH Chemicals, Poole, Dorset, U.K.). The guanidinium chloride was purified with activated charcoal (Norit GSX; Hopkin and Williams, Chadwell Heath, Essex, U.K.). Chondroitinase ABC and endo-β-D-galactosidase (keratanase) were obtained from Miles Laboratories (Slough, Berks., U.K.). Trypsin (diphenylcarbamoyl chloride-treated) (EC 3.4.21.4), testicular hyaluronidase (EC 3.2.1.35), 2-methylmaleic anhydride, 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, disodium EDTA, benzamidine hydrochloride, carbazole, Coomassie Brilliant Blue G and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Sepharose gels and Sephacryl S-300 were obtained from Pharmacia (Uppsala, Sweden). Oligosaccharides of hyaluronate were prepared by partial digestion with testicular hyaluronidase as described previously (Hascall & Heinegård, 1974; Hardingham, 1979).

General analytical procedures

Uronic acid was determined by an automated procedure (Heinegård, 1973) of the modified carbazole reaction (Bitter & Muir, 1962), with glucuronolactone as standard. Radioimmunoassays for binding region and link protein were as previously described (Ratcliffe & Hardingham, 1983).

Preparation of hyaluronate-binding proteins

Proteoglycan aggregates were extracted from pig laryngeal cartilage in 4M-guanidinium chloride/0.05M-sodium acetate buffer, pH5.8, in the presence of proteinase inhibitors, and purified in a CsCl density gradient as described by Hardingham (1979). The purified freeze-dried proteoglycan aggregate preparation was dissolved in 0.1M-Tris/acetate buffer, pH7.3, and digested for 50min with chondroitinase ABC (3.5 units/g) followed by trypsin (2mg/g) for 6h (Heinegård & Hascall, 1974). In this digestion the chondroitinase does not cleave the hyaluronate between each point of binding region and link protein attachment. The digest was concentrated by partial freeze-drying to one-third volume and chromatographed on a column (780mm × 23mm) of Sepharose CL-6B in 0.5M-sodium acetate buffer, pH6.8, at 4°C (see Fig. 1). The protein-rich V_0 fractions were pooled, dialysed against water, freeze-dried and dissolved in 4M-guanidinium chloride/1mM-Na_2EDTA/0.05M-sodium acetate buffer, pH5.8, and chromatographed on a column (1400mm × 16mm) of Sephacryl S-300 eluted with the same buffer at 20°C (see Fig. 2). The eluate was monitored at 230nm, and the two protein peaks of binding region and link protein, which were partially resolved, were pooled separately, concentrated and rechromatographed on the same column. Binding region isolated and freeze-dried as the sodium salt was freely soluble in water; the concentration was determined from $A^{1\%}_{278} = 0.65$ (Perkins *et al.*, 1981). Trypsin-prepared link protein was not freely soluble in water and was kept in 4M-guanidinium chloride/1mM-Na_2EDTA/0.05M-sodium acetate buffer, pH5.8, and the concentration was determined in this buffer from $A^{1\%}_{278} = 1.40$ (Tang *et al.*, 1979).

Link protein was also prepared without trypsin digestion from purified proteoglycan aggregate (A1) by fractionation in a CsCl density gradient under dissociative conditions to give a low-density protein-rich fraction (A1D3) (Hardingham, 1979), which was further purified by gel chromatography on Sephacryl S-300 in 4M-guanidinium chloride (Hardingham *et al.*, 1981).

Digestion of proteoglycan binding region with endo-β-D-galactosidase (keratanase)

Purified binding region (13.35mg/ml) was digested at 37°C for 24h in 0.1M-Tris/acetate buffer, pH7.3, in the presence of proteinase inhibitors with 0.01 unit of keratanase/mg (Oike *et al.*, 1980). The digest was dialysed against water and freeze-dried.

Preparation of link protein bound to hyaluronate oligosaccharides (HA_{10-16})

Link protein (200μl of 5.85mg/ml) was mixed with HA_{10-16} (146μg) in 4M-guanidinium chloride and dialysed against 1M-NaCl/0.01M-Mes buffer, pH7.0, in dialysis tubing previously heated at 95°C for 48h to decrease the porosity (Callanan *et al.*, 1957).

Preparation of the binding region–link protein–$HA_{\sim 34}$ complex

The two protein peaks of binding region and link protein chromatographed on Sephacryl S-300 in dissociative conditions (4M-guanidinium chloride) were pooled in equimolar amounts, dialysed against water and freeze-dried. The proteins were dissolved in 4M-guanidinium chloride/1mM-Na_2EDTA/0.05M-sodium acetate buffer, pH5.8 (7.4mg/ml), and oligosaccharides ($HA_{\sim 34}$) were added (with a molar excess of 25%). The mixture was dialysed against phosphate-buffered saline (0.123M-NaCl/0.014M-sodium phosphate buffer, pH7.2) and kept at 4°C.

Chemical modification of link protein with 2-methylmaleic (citraconic) anhydride

To link protein (340μl of 5.85mg/ml) in 4M-guanidinium chloride/0.014M-Na_2HPO_4, pH8.4, were added, at 30min intervals, four 2μl portions of 2-methylmaleic anhydride. The solution was stirred and maintained at pH8.4 by addition of 0.5M-NaOH with a pH-stat (Radiometer). The modified protein was then dialysed against several changes of water that had been adjusted to pH8.5 with 0.1M-NH_3. After this extensive dialysis, the sample was concentrated and dialysed against phosphate-buffered saline and stored at −20°C.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out on slab gels at several different polyacrylamide concentrations essentially as described by Fairbanks *et al.* (1971). Protein bands were detected by staining with Coomassie Brilliant Blue G (Blakesley & Boezi, 1977) and the gels were scanned with a Locarte densitometer. Molecular-mass calibrations were made with globular protein standards (M_r 12300–78000) or with cross-linked protein standards (M_r 14300–71500), both from BDH Chemicals.

Carbohydrate analysis

The content and molar ratio of galactosamine to glucosamine in the preparations was determined after hydrolysis in 8M-HCl under N_2 for 3h at 95°C by chromatography on a Locarte amino acid analyser (Hardingham *et al.*, 1976). Neutral sugar analysis was carried out by Dr. A. K. Allen (Charing Cross Hospital Medical School, London, U.K.) by the procedure of Chambers & Clamp (1971) with mannitol as internal standard. Sialic acid was determined by the method of Oegama & Cooper (1983).

Analytical-ultracentrifuge studies

M_r values and sedimentation coefficients were determined at 20°C in an MSE Centriscan analytical ultracentrifuge with a six-place analytical rotor, single-sector quartz cells and 20mm centre-pieces. The ultracentrifuge was equipped with absorption and schlieren detection systems.

The M_r were determined by sedimentation-equilibrium ultracentrifugation. The gradient of solute formed in the cell was detected with the u.v. scanner (280nm filter) after a run of 24h or 36h with a short column (3mm) at low speed (8000–15000rev./min) (Coates, 1970) and analysed as described in MSE Technical Publication no. 73. Values for the weight-average M_r at each solute concentration (c) were determined from linear regression of plots of ln(absorbance) or ln$c$ versus r^2. The M_r values were calculated by assuming the partial specific volumes to be binding region, 0.66ml/g, link protein, 0.73ml/g, and complex, 0.68ml/g (Perkins *et al.*, 1981).

For the determination of sedimentation coefficients, the samples were centrifuged at 40000 rev./min, and the detection was made with the schlieren system (binding region and complex) or u.v. absorption (link protein). Results were analysed as described in MSE Technical Publication no. 73.

Results

The preparations of binding region (BR) and link protein (LP-T) from chondroitinase ABC and trypsin digestion of aggregated proteoglycan were isolated by gel chromatography on Sepharose 6B-CL under associative conditions (Fig. 1) followed by separation on Sephacryl S-300 under dissociative conditions in 4M-guanidinium chloride (Fig. 2). Fractions containing purified binding region and link protein migrated as single major bands on SDS/polyacrylamide-gel electrophoresis, and specific radioimmunoassays (Ratcliffe & Hardingham, 1983) showed that there was minimal cross-contamination between the two preparations (less than 1%, w/w). Link protein was also prepared from proteoglycan aggregates without trypsin digestion by density-gradient centrifugation and chromatography on Sephacryl S-300 (Hardingham *et al.*, 1981). SDS/polyacrylamide-gel electrophoresis showed that the native link-protein preparation from pig laryngeal cartilage was composed of approx. 90% of high-M_r form and

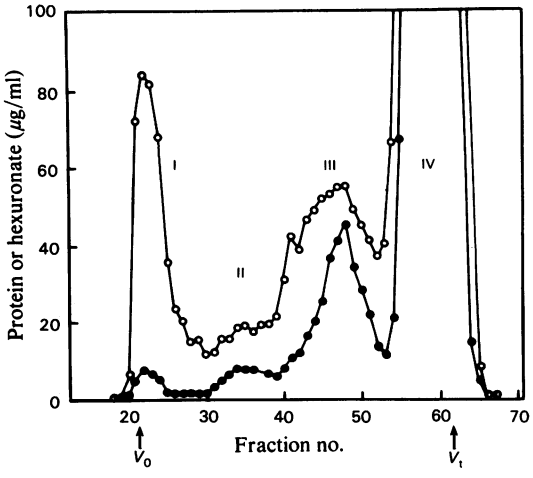

Fig. 1. *Gel chromatography on Sepharose 6B-CL of the chondroitinase ABC and trypsin digest of proteoglycan aggregate*

Proteoglycan aggregate was digested with chondroitinase ABC and trypsin as described in the Materials and methods section and chromatographed on a column (780mm × 23mm) of Sepharose 6B-CL in 0.5M-sodium acetate buffer, pH6.8, at 4°C. Fractions (5ml) were collected and the uronic acid (●) and protein (○) contents were determined. The V_0 fractions (I) were pooled, dialysed and freeze-dried. Analysis showed peak II to be rich in keratan sulphate, peak III to contain residual chondroitin sulphate attached to peptide and peak IV to contain digest products including chondroitin sulphate disaccharides (D. G. Dunham & T. E. Hardingham, unpublished work).

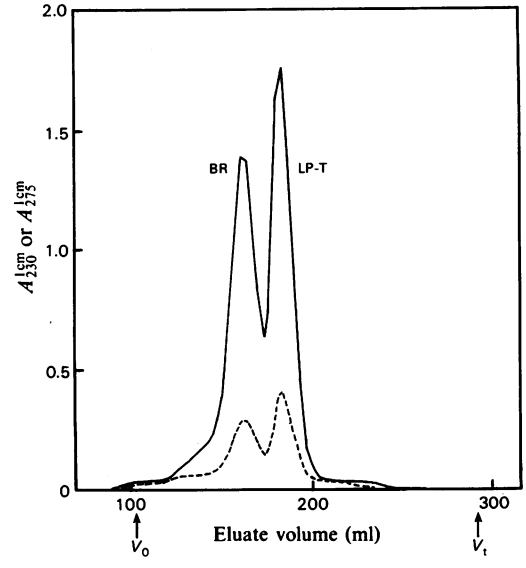

Fig. 2. *Gel chromatography on Sephacryl S-300 in 4M-guanidinium chloride of the binding region–link protein–hyaluronate complex isolated from the experiment shown in Fig. 1*

The sample was dissolved in 4M-guanidinium chloride/0.01M-Na_2EDTA/0.05M-sodium acetate buffer, pH5.8, and chromatographed on a column (1400mm × 16mm) of Sephacryl S-300 eluted in the same buffer at 20°C. The eluate was monitored at 230nm (——) and at 275nm (------). Key: BR, binding region; LP-T, link protein (trypsin-prepared).

approx. 10% of low-M_r form, whereas the trypsin-prepared link protein appeared slightly smaller than the low-M_r form, as reported by Baker & Caterson (1979).

Interaction with hyaluronate

It was shown previously that trypsin-prepared link protein (LP-T) showed properties of solubility and aggregate stabilization that were similar to those of the native link-protein preparation (LP) (Hardingham *et al.*, 1981). Interaction experiments were therefore used to test if isolated binding region also retained its native properties (Fig. 3). A sample of the purified binding region was mixed with hyaluronate and chromatographed in 1M-NaCl/0.01M-Mes buffer, pH7.0, on a column of Sephacryl S-300 at 20°C (Fig. 3*a*). It showed strong interaction under these conditions and was eluted in the void volume of the column, whereas in the absence of hyaluronate it was retarded by the column ($K_{av.}$ 0.31) (Fig. 3*a*). Interaction with hyaluronate was also shown to be reversible, as the addition of oligosaccharides of hyaluronate ($HA_{\sim 34}$) before chromatography released binding region, which formed an additional retarded protein peak (Fig. 3*b*). These results showed that the purified binding region retained its ability to interact with hyaluronate. Parallel experiments with the link-protein preparations showed similar properties of interaction with hyaluronate (Fig. 3*d*) and competition by oligosaccharides (Fig. 3*e*). When binding region was allowed to interact together with native link protein and hyaluronate and then chromatographed, they formed a single peak at the void volume of the column, and radioimmunoassays showed this to contain both binding region (results not shown) and link protein (Fig. 3*f*). The antigenic sites on link protein were also shown to be 95% masked, as they required heat treatment in SDS for full detection (Fig. 3*f*). This was similar to the masking of link-protein antigenic sites observed in the intact proteoglycan aggregate (Ratcliffe & Hardingham, 1983). The complex formed between binding region, link protein and hyaluronate was also found to be undissociated by oligosaccharides of hyaluronate (HA_{10-16}) (results not

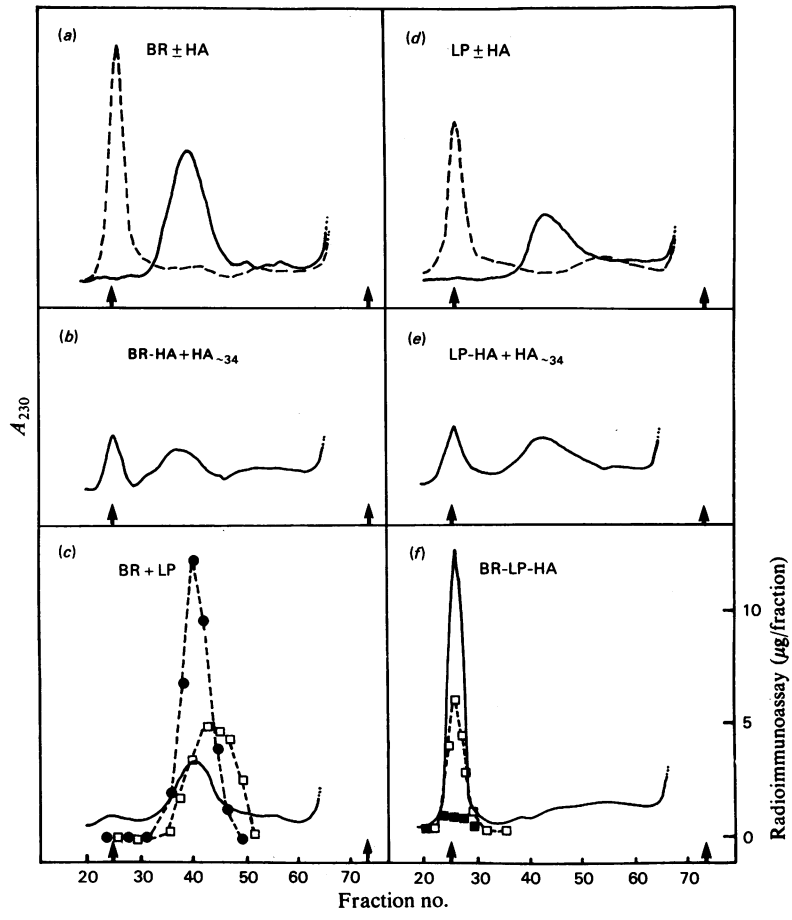

Fig. 3. *Interaction of proteoglycan binding region (BR) with link protein (LP) and with hyaluronate (HA)*
Samples of binding region (72 μg), link protein (50 μg) and hyaluronate (23 μg) were chromatographed on a column (750 mm × 8 mm) of Sephacryl S-300 in 1 M-NaCl/0.01 M-Mes buffer, pH 7.0, at 20°C. All components were mixed in up to 500 μl of 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8, and then diluted 10-fold in five steps over 8 h with 0.05 M-sodium acetate buffer, pH 5.8. $HA_{\sim 34}$ oligosaccharides (23 μg) were added to (*b*) and (*e*) after dilution and 1 h before chromatography. (*a*) Binding region with (----) or without (——) hyaluronate; (*b*) $HA_{\sim 34}$ added to binding region already bound to hyaluronate; (*c*) binding region with link protein; (*d*) link protein with (----) or without (——) hyaluronate; (*e*) $HA_{\sim 34}$ added to link protein already bound to hyaluronate; (*f*) binding region with link protein and hyaluronate. Graphs show the recorder trace from the column eluate passed through a flow cell monitored at 230 nm. Fractions (0.5 ml) were collected and radioimmunoassay was carried out on some samples for link protein with (□) and without (■) treatment with 0.025% SDS and for total binding region (●) as described by Ratcliffe & Hardingham (1983).

shown). It therefore showed properties of stabilization by link protein similar to that in the native proteoglycan aggregate (Hardingham, 1979)

Binding region and link protein chromatographed together, but without hyaluronate did not show strong interaction (Fig. 3*c*). They were eluted as a retarded protein peak ($K_{av.}$ 0.3) in which the two proteins overlapped but were not co-eluted (as shown by radioimmunoassay) (Fig. 3*c*), and the link-protein antigenic determinants showed no masking (results not shown).

Determination of M_r values

As two species were present in the native link-protein preparation, determinations of M_r were carried out on the trypsin-prepared link protein. Preliminary estimates of M_r were obtained by electrophoresis in SDS/polyacrylamide gels of differing porosity by comparison with standard proteins. Link protein gave a tight band with $M_{r(app.)}$ 40000, which did not vary, but binding region showed a broader band with an apparent

average $M_{r(app.)}$ that decreased down to 70000 (Fig. 4) as the gel porosity increased. Digestion of binding region with keratanase in the presence of proteinase inhibitors gave a sharper protein band on electrophoresis, and the average $M_{r(app.)}$ estimate was decreased to 60000 (Fig. 4).

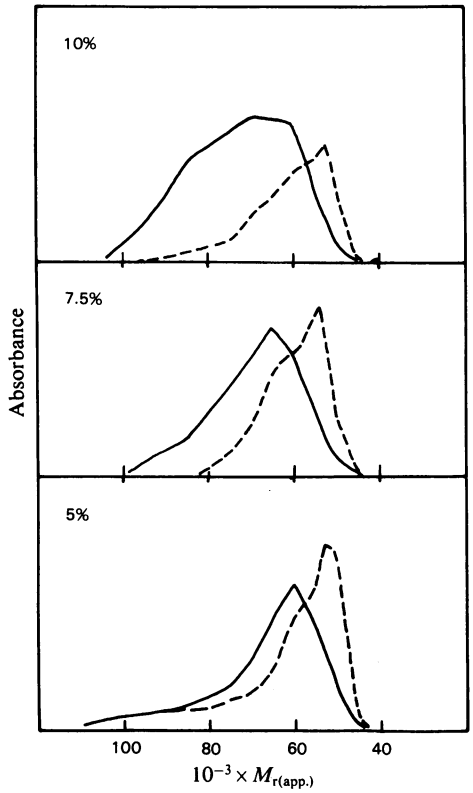

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of proteoglycan binding region before and after digestion with keratanase*

Samples of binding region before (——) and after (– – – –) digestion with keratanase were electrophoresed on slab gels of three porosities as described in the Materials and methods section. The gels were stained with Coomassie Brilliant Blue G and scanned with a densitometer. The apparent M_r values were calculated in comparison with globular protein standards.

Carbohydrate analysis of the preparations showed link protein to be of low carbohydrate content (less than 4%, w/w), but binding region showed a high content (25%, w/w) and a composition suggesting both *N*-linked complex oligosaccharides and *O*-linked keratan sulphate chains to be present. This was supported by the results from keratanase digestion, which showed the total glucosamine content to be decreased by 25% and galactose by 28%, whereas mannose was unaffected (Table 1). The response of keratanase-digested binding region in the radioimmunoassay to binding region remained unchanged, but in a radioimmunoassay to keratan sulphate with a monoclonal antibody (Caterson *et al.*, 1983) it was found to be decreased by 47%. The enzyme had thus removed at least part of the keratan sulphate chains. Experiments similar to those in Fig. 3 showed that the interaction properties of binding region with hyaluronate and link protein were unaffected by this treatment (results not shown).

Further M_r determinations were therefore carried out on the trypsin-prepared link protein and on binding region before and after keratanase digestion.

Analytical ultracentrifugation

Link protein. When solutions of link protein were dialysed from 4M-guanidinium chloride into phosphate-buffered saline they showed high sedimentation coefficients (Table 2), which appeared to result from self-association into oligomeric species (Tang *et al.*, 1979; Hardingham *et al.*, 1981). These solutions were therefore inappropriate for M_r determinations. Preliminary experiments suggested that the self-association was inhibited in the presence of hyluronate oligosaccharides (D. G. Dunham & T. E. Hardingham, unpublished work). Addition of oligosaccharides (HA_{10-16}) before dialysis into 1M-NaCl was found to decrease the sedimentation coefficient from 9.3S to 4.2S (Table 2 and Fig. 5). However, sedimentation-equilibrium analysis suggested this preparation to consist mainly of dimers, as the average M_r was 77800 ± 2700 (Fig. 6).

Table 1. *Carbohydrate composition of preparations from cartilage proteoglycan*

Abbreviation: N.D., not determined.

	Binding region (% dry wt.)	Keratanase-digested binding region (% dry wt.)	Link protein (trypsin-prepared) (% dry wt.)
Mannose	2.3	2.2	0.2
Galactose	9.1	6.5	0.3
N-Acetylglucosamine	9.3	7.0	2.4
N-Acetylgalactosamine	0.9	1.1	0.8
Sialic acid	0.5	0.5	N.D.

Table 2. *Physical properties of preparations from cartilage proteoglycan*

	$10^{-3} \times M_r$	Sedimentation coefficient (S)	Gel chromatography (Sephacryl S-300/1 M-NaCl $K_{av.}$)	Molecular structures
Binding region	66.5 ± 5.5 (70*)	3.4 ± 0.1	0.31	
Keratanase-digested binding region	60.8 ± 4.9 (60*)	3.3 ± 0.1	0.39	
Link protein	> 100 (40*)	~9.3		
Link protein + HA_{10-16}	77.8 ± 2.7	4.2 ± 0.3	0.32	
Link protein (2-methylmaleyl-substituted)	41.7 ± 2.5 (40*)	2.5 ± 0.06	0.59	
Complex: binding region–link protein–$HA_{\sim 34}$	133.5 ± 6.7	5.5 ± 0.1	0.22	

* $M_{r(app.)}$ estimates from SDS/polyacrylamide-gel electrophoresis.

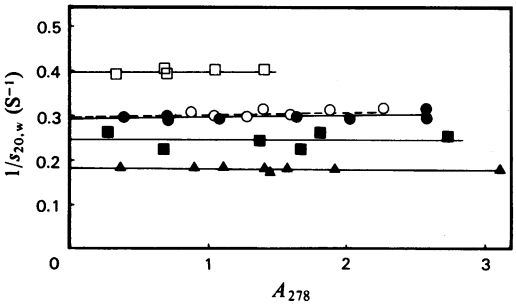

Fig. 5. *Determination of sedimentation coefficients*
Samples of binding region before (●) and after (○) keratanase digestions, 2-methylmaleylated link protein (□) and binding region–link protein–$HA_{\sim 34}$ complex (▲) in phosphate-buffered saline and link protein + HA_{10-16} (■) in 1 M-NaCl/0.01 M-Mes buffer pH 8.0, were centrifuged at 40000 rev./min in the analytical ultracentrifuge as described in the Materials and methods section. The cells were monitored at timed intervals with the use of schlieren optics, except for the link-protein samples, which were analysed by u.v. absorbance. Concentrations were determined from the absorbance at 275 nm of dilutions of the samples. Results, calculated as described in the Materials and methods section, did not show strong concentration-dependence, and values at zero concentration are mean values for each sample, except for binding region, where linear regression was used to discriminate between the determinations for the normal and keratanase-digested sample.

It was found possible to obtain monomeric link protein in low-ionic-strength buffers (phosphate-buffered saline) by treating it with 2-methylmaleic anhydride to modify lysine amino groups. It then gave a sedimentation coefficient of 2.5S (Fig. 5) and an M_r of 41700 ± 2500 (Fig. 6), which was similar to the determination of M_r under dissociative conditions in 4 M-guanidinium chloride (results not shown).

Binding region. Binding region before or after keratanase digestion was freely soluble in phosphate-buffered saline and showed a small difference in sedimentation coefficient (3.4 ± 0.1 S and 3.3 ± 0.1 S respectively) (Fig. 5). Sedimentation-equilibrium analysis showed the M_r to be decreased by keratanase digestion from 66500 ± 5500 to 60800 ± 4900 (Fig. 6 and Table 2). Although there was some overlap of the experimental points, a decrease in M_r of 5700 was close to the loss (4700) calculated from the change in glucosamine content, assuming it to be part of the keratan sulphate repeat disaccharide units removed by the enzyme.

Binding region–link protein–$HA_{\sim 34}$ complex. Mixing in 4 M-guanidinium chloride followed by dialysis formed a ternary complex between binding region (BR), link protein (LP-T) and oligosaccharides of hyaluronate ($HA_{\sim 34}$). Gel chromatography of a sample on Sephacryl S-300 showed a single protein peak ($K_{av.}$ 0.22) without evidence of free binding region or link-protein components. It also formed a single boundary in sedimentation-velocity analysis with a sedimentation coefficient, 5.5 ± 0.1 S (Fig. 5), that was larger than that of free binding region or link protein. Sedimentation-equilibrium analysis showed a linear correlation of $\ln c$ with r^2, showing a single molecular species to be formed. The average of the M_r determinations between 0.87 mg/ml and 3.43

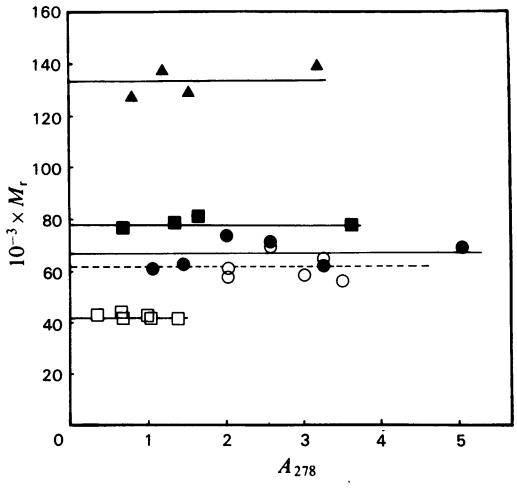

Fig. 6. *Determination of M_r values by sedimentation-equilibrium analysis*

Samples were dialysed against phosphate-buffered saline and centrifuged for 24–36h at 20°C. Binding region before (●) and after (○) keratanase digestion was centrifuged at 8500rev./min, 2-methylmaleyl-substituted link protein (□) at 15000rev./min and binding region–link protein–$HA_{\sim 34}$ complex (▲) at 8000rev./min. Link protein + HA_{10-16} (■) was studied in 1M-NaCl/0.01M-Mes buffer, pH8.0, at 15000rev./min. Concentrations were determined from absorbance measurements at 275nm of dilutions of the samples. Results (Table 2) were calculated as described in the Materials and methods section. Values at zero concentration are the means of the determinations at different dilutions for each sample.

mg/ml was 133500+6700 (Fig. 6 and Table 2) (linear regression gave M_r 126600). This was in reasonable agreement with a 1:1:1 complex of binding region, link protein and $HA_{\sim 34}$, although it was rather higher than the calculated value (114000). With the radioimmunoassay the complex showed 95% masking of link-protein antigenic determinants. The physical and immunochemical properties were thus in good agreement with the interaction of three components in equimolar amounts to form a structure similar to that occurring in native proteoglycan aggregates.

Discussion

The link protein isolated after trypsin digestion of proteoglycan aggregates showed properties of self-association into oligomeric species similar to that of the native link protein (Tang *et al.*, 1979; Hardingham *et al.*, 1981). The self-association was partially inhibited in the presence of oligosaccharides HA_{10-16}, when it formed dimers. This suggested a model of self-association in which link protein has two sites for self-interaction, one of which is abolished when link protein binds to hyaluronate (see Table 2). Thus, when it interacts with a short hyaluronate oligosaccharide, which is univalent (HA_{10-16}), it can only form dimers. The absence of self-association in the assembled binding region–link protein–$HA_{\sim 34}$ complex suggests that the presence of binding region and hyaluronate blocks both sites of self-association. This is quite compatible with the evidence of the preferential masking of the antigenic sites of link protein compared with those of binding region when both are bound in the complex, which suggests that link protein may be rather buried in this structure.

Reaction of link protein with 2-methylmaleic anhydride was previously reported to increase its solubility (Wieslander & Heinegård, 1980). The present results show that this is achieved by abolishing self-association, producing a monomeric preparation of M_r 41700. The extent of substitution of free amino residues was not determined, but if it were 50% of the total lysine residues the M_r of the untreated protein would be 40450. For link protein the M_r determined by sedimentation-equilibrium experiments was therefore in good agreement with the estimate from SDS/polyacrylamide-gel electrophoresis.

The binding region of proteoglycan prepared by trypsin digestion of aggregate was found to contain keratan sulphate, as reported by Heinegård & Hascall (1974), and this was partially removed by keratanase digestion. Examination of the carbohydrate composition of binding region and its M_r (66800) suggests that the carbohydrate was distributed in relatively few chains. From the *N*-linked oligosaccharide structures found in other proteoglycans (Lohmander *et al.*, 1980) and the structure of keratan sulphate (Hascall & Hascall, 1980), calculations suggest that on average only three chains of each are present per molecule of binding region.

The M_r of the binding region determined by sedimentation-equilibrium analysis (M_r 66500) was smaller than that determined by SDS/polyacrylamide-gel electrophoresis ($M_{r(app.)}$ 70000), but these were in much better agreement when the electrophoresis was calibrated with globular-protein standards than when calibrated with polymerized-protein standards as published previously ($M_{r(app.)}$ 83000; Perkins *et al.*, 1981). The M_r of the keratanase-digested binding region (60500) was in good agreement with the estimate from SDS/polyacrylamide-gel electrophoresis. After removal of some keratan sulphate, the electrophoresis of binding region was thus even more comparable with the globular-protein standards. From the results the M_r of the protein in the binding-region preparations was 49000. The protein domain of

proteoglycan that is involved in aggregation is thus not much larger in size than link protein and would account for only 14% of the total amino acid sequence, as the reported M_r of the newly synthesized protein core is 340000 (Upholt *et al.*, 1979; Treadwell *et al.*, 1980).

The complex formed between binding region, link protein and $HA_{\sim 34}$ showed properties entirely consistent with an assembled structure similar to that involved in aggregation. It contained a single species whose apparent size, M_r and antigenic properties were in good agreement with a 1:1:1 complex. The properties of the complex are comparable with the results reported by Faltz *et al.* (1979), who prepared a binding region–link protein–hyaluronate complex directly from proteoglycan aggregates after trypsin and chondroitinase ABC digestion. In their study the digestion with chondroitinase ABC was carried to completion in order to cleave the hyaluronate between each binding region–link protein complex. The size of a predominant hyaluronate fragment recovered was $HA_{\sim 41}$, showing that this was protected from further digestion by being part of the complex, and by gel chromatography the complex had $M_{r(app.)}$ 112000.

The structure and stability of proteoglycan aggregates is dependent on the precise assembly of the binding region–link protein–hyaluronate complex. The properties of the preparations described in the present paper suggest that they will prove appropriate for further detailed investigation of the molecular interactions involved in aggregation.

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