Biochem. J. (1979) **179**, 35–45
Printed in Great Britain

Immunochemical Analysis of Cartilage Proteoglycans

ANTIGENIC DETERMINANTS OF SUBSTRUCTURES

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(*Received* 11 *September* 1978)

Antibodies were raised in rabbits by injection of cartilage proteoglycan monomers, isolated hyaluronic acid-binding region, polysaccharide–peptides prepared by trypsin digestion of proteoglycans and link-protein. The rabbits injected with the proteoglycan monomers made antibodies reacting with the intact proteoglycan. The antiserum contained antibodies specific for, and also reacting with, the isolated hyaluronic acid-binding region and the keratan sulphate-rich region. In addition there were probably antibodies reacting with other structures of the proteoglycan monomer. When isolated hyaluronic acid-binding region was used for immunization the antibodies obtained reacted specifically with the hyaluronic acid-binding region. The antibodies obtained from rabbits immunized with the polysaccharide-peptides reacted with the proteoglycan monomers and showed a reaction identical with that of the chondroitin sulphate–peptides isolated after trypsin digestion of proteoglycans. The antibodies prepared with the link-protein as the antigen reacted only with the link-protein and not with any preparation from the proteoglycan monomer. Neither did any of the antisera raised against the proteoglycan monomer or its substructures react with the link-protein. Separately it was shown that the peptide 'maps' prepared from trypsin digests of the link-protein and the hyaluronic acid-binding region were different. Therefore it appears that the link-protein is not structurally related to the proteoglycan or the hyaluronic acid-binding region. Digestion of proteoglycan monomers or isolated hyaluronic acid-binding region with trypsin did not destroy the antigenic sites of the hyaluronic acid-binding region. In contrast trypsin digests of previously reduced and alkylated preparations did not react with the anti-(hyaluronic acid-binding region). The trypsin digests, however, reacted with both the antibodies directed against the chondroitin sulphate–peptides and those against the keratan sulphate–peptides. Trypsin digestion of the link-proteins destroyed the antigenic site and the reactivity with the antibodies. By combining immunoassay of proteoglycan preparations before and after trypsin digestion it is feasible to quantitatively determine its substructures by using the antisera described above.

Three substructures of the central protein core of the proteoglycans have been described (Heinegård & Axelsson, 1977). The hyaluronic acid-binding region at one end of the protein core contains few or no polysaccharide side chains and can interact specifically with hyaluronic acid and probably also with link-protein (Heinegård & Hascall, 1974*a*, 1978; Baker & Caterson, 1978). This region contains about one-third of the total protein in the proteoglycan. At the other end of the core, containing on average about half of the protein, is the chondroitin sulphate-rich region (Heinegård, 1977; Heinegård & Hascall, 1974*b*), which contains a very large number of chondroitin sulphate side chains attached via their reducing end to serine residues in the protein. The chondroitin sulphate chains are probably grouped in clusters with few amino acids separating the chains. Longer sequences of amino acids probably separate the clusters (Heinegård & Hascall, 1974*b*; Thyberg *et al.*, 1975). The keratan sulphate-rich region is located between the two regions described above (Heinegård & Axelsson, 1977). The protein in this region constitutes about one-tenth of the total in the proteoglycan and has a unique amino acid composition The closely spaced keratan sulphate chains are attached to serine and threonine residues.

The chemically different substructures of the proteoglycans could provide different antigenic sites, and immunochemical studies have indicated that more than one antigenic site is probably present in the proteoglycan (Loewi & Muir, 1965; Tsiganos & Muir, 1969; Keiser & De Vito, 1974). Most of these investigations, however, were done before the purification methods at present available had been introduced. It is therefore possible that the results obtained were influenced by the presence of contaminating

non-proteoglycan proteins. The present investigation was undertaken to characterize antibodies prepared to intact proteoglycans or to substructures of proteoglycans isolated after chemical cleavage.

A preliminary account of some of the results in the present paper have been reported elsewhere (Wieslander & Heinegård, 1977).

Materials and Methods

Bovine nasal cartilage was obtained at the local slaughterhouse immediately after the animals were killed and separated from mucosa and perichondrium. Chemicals used were of the best grade commercially available. The agarose used was obtained from Litex (Glostrup, Denmark).

Preparation of proteoglycans and substructures

Proteoglycans were prepared essentially as described previously (Hascall & Heinegård, 1974). Cartilage slices were extracted under dissociating conditions at 4°C for 20h with 4M-guanidine hydrochloride, 0.05M-sodium acetate, pH5.8, containing the following proteinase inhibitors (Oegema *et al.*, 1975): benzamidine hydrochloride (0.005M), ε-aminohexanoic acid (0.1M) and EDTA (0.01M). Proteoglycans in the extract were reassociated when the guanidine hydrochloride concentration was decreased by dialysis against 10vol. of 0.05M-sodium acetate, pH5.8, containing the proteinase inhibitors. The proteoglycan aggregate (A1) preparation was then isolated by CsCl-density-gradient centrifugation as described previously (Hascall & Heinegård, 1974; Heinegård, 1972). In some instances the proteoglycan monomers were prepared from the aggregate (A1) fraction by density-gradient centrifugation in CsCl/4M-guanidine hydrochloride to prepare the A1-D1 fraction (Hascall & Heinegård, 1974; Heinegård, 1972). Alternatively the link-proteins were removed from the proteoglycan aggregates by chromatography on Sephadex G-200 eluted with 4M-guanidine hydrochloride 0.05M-sodium acetate, pH5.8. The proteoglycan monomers and the hyaluronic acid were recovered from the void-volume peak, whereas the link-proteins were retarded (Heinegård & Hascall, 1974*a*).

All proteoglycan preparations used for immunization were fractionated on the preparative Sephadex G-200 column eluted with 4M-guanidine hydrochloride 0.05M-sodium acetate, pH5.8, to remove any extraneous protein material that was included in the column. The proteoglycan, or fragments thereof, chromatographed in the void volume of the column. The hyaluronic acid-binding region and the link-protein were prepared from the A1 fraction as described previously (Heinegård & Axelsson, 1977). Essentially the aggregate preparation was digested with diphenylcarbamoyl chloride-treated trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and the digest was fractionated by associative CsCl-density-gradient centrifugation to yield a bottom fraction (A1-T-A1) containing chondroitin sulphate–peptides from the chondroitin sulphate-rich region and keratan sulphate–peptides from the keratan sulphate-rich region (Heinegård & Axelsson, 1977). The top fraction contained in addition to peptides a complex of hyaluronic acid–link-protein–hyaluronic acid-binding region (Heinegård & Axelsson, 1977; Heinegård & Hascall, 1974*a*). This complex was further purified by Sepharose 2B chromatography and was recovered as a component of very large size from near the void volume, whereas the peptides chromatographed in the total volume (Heinegård & Axelsson, 1977; Heinegård & Hascall, 1974*a*).

The hyaluronic acid-binding region and the link-protein were separated from each other and also from the hyaluronic acid by Sephadex G-200 chromatography in 4M-guanidine hydrochloride (Heinegård & Hascall, 1974*a*). The two protein preparations obtained were rechromatographed on the same column to ensure complete separation. The A1-T-A1 fraction was passed through the same column of Sephadex G-200 eluted with 4M-guanidine hydrochloride to remove any extraneous protein.

Isolation of the keratan sulphate-rich region and the chondroitin sulphate-rich region were carried out as described previously (Heinegård & Axelsson, 1977). Essentially the bottom fraction (A1-T-A1) from the associative CsCl density gradient of the trypsin-digested aggregates was recovered and digested with chondroitinase ABC. The digest was chromatographed on Sepharose 6B to separate two major protein peaks, the larger containing the keratan sulphate–peptides and the smaller containing the chondroitin sulphate oligosaccharide–peptides.

Enzymic digestion

Chondroitinase ABC (Miles Chemicals, Elkhart, IN, U.S.A.) digestion was carried out at 37°C for 5h in 0.1M-sodium acetate/0.1M-Tris/acetic acid, pH7.3, with 1 unit of enzyme/100 mg of sample. One unit of enzyme is the amount required to release 1 μmol of disaccharide/min at 37°C and pH8.0. Trypsin digestion was carried out with diphenylcarbamoyl chloride-treated trypsin in 0.1M-sodium phosphate buffer, pH7.6, for 4h at 37°C; 2–4mg of enzyme was used per 100mg of protein in the samples. Digestion with α-chymotrypsin (Koch–Light, Colnbrook, Bucks., U.K.) was performed at 37°C for 5h in 0.1M-sodium acetate/0.1M-Tris/acetic acid, pH7.3; 2–4mg of enzyme was used per 100mg of protein in the samples.

Reduction and alkylation

Reduction was carried out with less than 0.5mg of protein in 1ml of 4M-guanidine hydrochloride/0.05M-

Tris/HCl, pH7.6, containing 5mM-dithiothreitol at 37°C for 5h. Iodoacetic acid (3-fold excess) was added and the alkylation continued overnight in darkness at room temperature. The samples were then dialysed against water and freeze-dried.

Peptide 'mapping'

Reduced and alkylated preparations of hyaluronic acid-binding region and link-protein respectively were digested at 37°C for 4h with diphenylcarbamoyl chloride-treated trypsin (substrate/enzyme ratio, 1:100) in aq. 1% NH_4HCO_3 and then freeze-dried. Samples of the digests (usually containing 20–50μg of protein) were then electrophoresed and chromatographed on silica-coated thin-layer plates essentially as described by Bates *et al.* (1975).

Citraconoylation and maleoylation of the link-protein

The link-protein had to be citraconoylated (Atassi & Habeeb, 1972) to remain in solution during analytical procedures. A sample of link-protein (2mg) was suspended in 1ml of water and the pH was adjusted to 8.2, and four 2μl portions of citraconic anhydride were added to the stirred solution at 30min intervals. The pH was maintained by addition of 0.1M-NaOH with a pH-stat. The citraconoylated protein was then dialysed against water that had been adjusted to pH8.5 with NH_3 and freeze-dried.

Alternatively the link-proteins were maleoylated as described by Butler & Hartley (1972) to remain in solution. Link-protein (1mg) was dissolved in 400μl of 0.1M-$NaHCO_3$. The protein was allowed to react for 5min with maleic anhydride that was added to a final concentration of 0.01M. The pH was maintained at 8.5 as described above.

Preparation of antisera

Rabbits were injected subcutaneously in the neck and in some cases also in the foot-pads with emulsions of fraction A1-D1 (5mg), purified link-protein (0.7mg), fraction A1-T-A1 (25mg) rechromatographed on Sephadex G-200 or purified hyaluronic-acid-binding region (1.0mg); all antigens were in Freund's complete adjuvant mixed with an equal proportion of 0.9% NaCl. Booster doses with the same amount of antigen in Freund's incomplete adjuvant were given after 4 weeks and then every second week until the antibody titres were constant. The rabbits were bled 10–12 days after the last booster and then every 2 weeks.

The γ-globulins were purified by precipitation with octanoic acid, ion-exchange chromatography on DE-52 DEAE-cellulose and precipitation with $(NH_4)_2SO_4$ as described by Steinbuch & Audran (1969).

Preparations of antigens for immunoassay

In general samples containing large proportions of chondroitin sulphate, e.g. proteoglycans or proteolytic fragments containing the chondroitin sulphate chains, were digested with chondroitinase before immunoassay. Usually 2mg of proteoglycan was digested in 0.5ml of 0.1M-sodium acetate/0.1M-Tris/acetic acid, pH7.3, with about 0.02 unit of chondroitinase ABC for 4h at 37°C. The digest was then dialysed extensively against water and freeze-dried. The samples were dissolved in the buffer used for immunoassay before use at concentrations giving about 0.1mg of protein/ml.

Immunological methods

Quantitative determinations were carried out by the electroimmunoassay 'rocket' method of Laurell (1966). Electrophoreses were performed on glass plates (25cm×10cm) covered with 25ml of 1% agarose (Laurell, 1966). The buffer used was 0.024M-barbital, pH8.6, containing an appropriate volume (usually 0.5–2ml) of γ-globulin per plate. Samples (12μl) were added to the application wells at the side of the plate facing the cathode. The electrophoresis was run for 16h at 5V/cm, with cooling of the plate by running tap water. The plates were washed in 0.15M-NaCl containing 0.05% NaN_3 overnight, dried and stained in Coomassie Brilliant Blue G-250.

Cross-reactions between different antigens were studied by immunoelectromigration by the method of Grubb (1972). Agarose (1%) and 0.024M-barbital buffer, pH8.6, with the same amount of antibodies as for the electroimmunoassay procedure was used with one antigen in the slit and the other in the well. The plates were washed and stained as described above. Alternatively immunological identities were demonstrated by Ouchterlony (1962) double diffusion in 0.8% agarose.

Crossed immunoelectrophoresis by the method of Clarke & Freeman (1967) was carried out in 0.024M-barbital buffer, pH8.6, on 1% agarose. Electrophoresis in the first dimension was run on plates (5cm×5cm) for 10min at 20V/cm. Before the second-dimension run, antibodies were cast with the agarose and the samples were electrophoresed for 16h at 5V/cm.

Results and Discussion

The reactivity of the antisera against the antigens used for immunization was tested, primarily by using the 'rocket' method for electroimmunoassay (Laurell, 1966).

It appeared that the antiserum produced against the purified link-protein [anti-(link-protein)] gave a

strong reaction against citraconoylated or maleoylated link-protein as indicated by the precipitate rocket (Fig. 1*a*). The antiserum directed against the purified hyaluronic acid-binding region [anti-(hyaluronic acid-binding region)] also had a high titre for antibodies (Fig. 1*b*). The proteoglycan monomers yielded an antiserum [anti-(A1-D1 fraction)] with a relatively high titre for antibodies reacting with chondroitinase-digested proteoglycan monomers (Fig. 1*c*). These antibodies also reacted with intact proteoglycans, although the 'rockets' obtained were not clearly visible down to the starting well (results not shown), possibly owing to mobility of the complexes because of the large number of negatively charged groups in the molecules. The antiserum directed against the glycosaminoglycan peptides [anti-(A1-T-A1 fractions)] prepared after trypsin digestion of the aggregates did not react with intact fragments (A1-T-A1). When the chondroitin sulphate side chains had been removed by chondroitinase digestion, on the other hand, the peptides showed a relatively weak reactivity with the antibodies, indicating a relatively low titre (Fig. 1*d*). It was also shown (results not included) that none of the antisera reacted with bovine plasma proteins, indicating purity of the antigens.

Specificities of antisera

The four different antisera were tested for specificities. Intact proteoglycan monomer, chondroitinase ABC-digested proteoglycan monomers (core protein), keratan sulphate-rich region and chondroitin sulphate–peptides isolated from the chondroitin sulphate-rich region after trypsin digestion of proteoglycans, hyaluronic acid-binding region isolated after trypsin digestion of the aggregate and link-protein were electrophoresed in gels containing one of the four antibodies (Fig. 2). The anti-(A1-D1 fraction) antibodies reacted and gave precipitates ('rockets') with both the proteoglycan monomers and the core-protein preparations (Fig. 2*a*). The 'rocket' obtained with the isolated keratan sulphate-rich region was high, although the precipitate did not extend to the starting point. It is possible that the highly negatively charged antigens have caused the precipitate to move. Similar results with highly charged molecules have

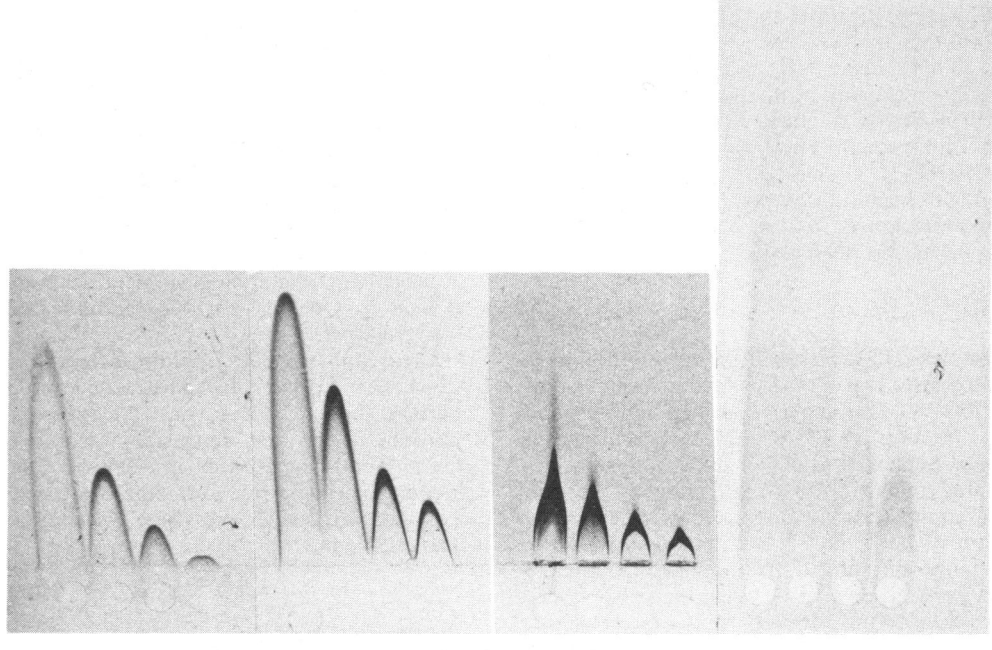


Fig. 1. *Electroimmunoassay of the link protein against anti-(link-protein) (a), hyaluronic acid-binding region against anti-(hyaluronic acid-binding region) (b), proteoglycan monomer (A1-D1-CB-6B1) against anti-(A1-D1 fraction) (c) and polysaccharide–peptides prepared from trypsin digests of proteoglycan aggregates (A1-T-A1-CB) against anti-(A1-T-A1 fraction) (d)*

Concentrations of antigens were (from left to right within *a–d*) 200, 100, 50 and 25 μg/ml.

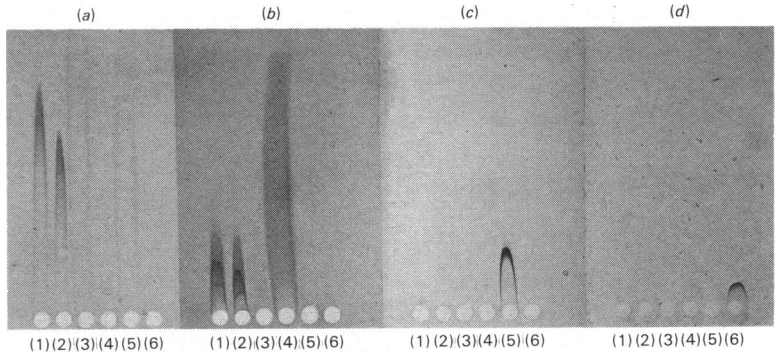


Fig. 2. *Electroimmunoassay of proteoglycan monomer (A1-D1) (1), core protein (A1-D1-CB-6B1) (2), keratan sulphate-rich region (A1-T-A1-CB-6B1) (3), chondroitin sulphate–peptides prepared from trypsin digest of proteoglycan aggregates (A1-T-A1-CB-6B2) (4), hyaluronic acid-binding region (A1-T-A3-2B1-G200-2) (5) and link-protein (A1-T-A3-2B1-G200-3) (6) against anti-(A1-D1 fraction) (a), anti-(A1-T-A1 fraction) (b), anti-(hyaluronic acid-binding region) (c) and anti-(link-protein) (d)*
All concentrations were 50 μg/ml.

been observed in other systems (Wieslander *et al.*, 1977). The isolated hyaluronic acid-binding region also gave a high 'rocket', indicating a high proportion of antigen to antibodies. Neither the link-protein nor, somewhat surprisingly, the chondroitin sulphate–peptides that had been isolated from chondroitinase-digested A1-T-A1 fragments (A1-T-A1-CB-6B2) reacted with the antibodies to form a precipitate. The results show that the proteoglycan monomers give rise to antibodies directed against more than one site on the molecules, and corroborating evidence is discussed below.

The antibodies raised against the A1-T-A1 fraction, which contains chondroitin sulphate–peptides and the keratan sulphate-rich-region peptide, reacted only with antigens containing sequences of the chondroitin sulphate-rich region (Fig. 2*b*). 'Rockets' were obtained with the monomer (A1-D1), the core protein and chondroitin sulphate–peptides isolated from chondroitinase digests of the A1-T-A1 fraction (A1-T-A1-CB-6B2). None of the other antigens tested reacted to give a visible 'rocket'. In particular the isolated keratan sulphate-rich region did not react with the antibodies although the material used for immunization contained this fragment. The antibodies raised against the isolated hyaluronic acid-binding region, as expected, reacted with this antigen (Fig. 2*c*). 'Rockets' were also obtained both with the A1-D1 fragment and the core protein, which contain the hyaluronic acid-binding region structure, although these 'rockets' were very high and faint, perhaps indicating a low proportion of antibodies to reacting antigen. Another explanation could be that the large number of negative charges on these antigens interfered with the precipitation. Trypsin digests of both the A1-D1 fragment and the core protein gave strong 'rockets' of expected height (see Fig. 8), indicating that the trypsin digestion had liberated the antigenic peptides. None of the other antigens tested gave any precipitate.

The antibodies raised against the link-protein isolated from trypsin digests of aggregates reacted only with the link-protein. The antigen, however, either had to be citraconoylated or maleoylated to remain in solution when tested. This modification did not abolish reactivity.

The homogeneity of the antigens were tested by using crossed immunoelectrophoresis by the method of Clarke & Freeman (1967). Link-protein, hyaluronic acid-binding region, core protein, keratan sulphate-rich peptides and peptides from the chondroitin sulphate-rich region (A1-T-A1-CB-6B2) were electrophoresed on agarose gels in one dimension and then electrophoresed in the other dimension on agarose gels containing anti-(link-protein), anti-(hyaluronic acid-binding region), anti-(A1-D1 fraction) and anti-(A1-T-A1 fraction) (polysaccharide–peptides obtained from trypsin digests of proteoglycan aggregates) respectively. In addition the core preparation was also analysed with anti-(A1-T-A1 fraction) as the antibody (Fig. 3). The link-protein and the keratan sulphate-rich peptide preparations gave fairly sharp well-defined peaks, whereas the other samples gave broader peaks, probably due to polydispersity. The core preparation appeared to yield two components (precipitation lines) with anti-(A1-D1 fraction), whereas only one component was visible with anti-(A1-T-A1 fraction). It is possible that the two components are related to the two components observed on polyacrylamide–agarose electrophoresis of proteoglycan monomers (Mason & Mayer, 1973; Stanescu *et al.*, 1977).

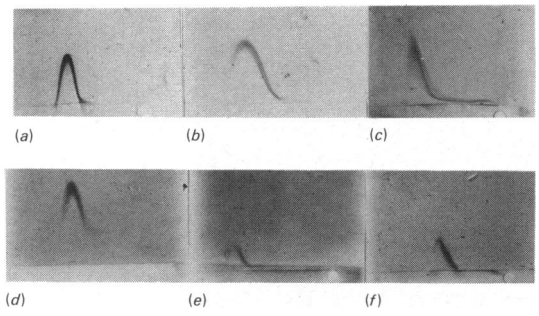


Fig. 3. *Crossed immunoelectrophoresis of link-protein against anti-(link-protein) (a), hyaluronic acid-binding region against anti-(hyaluronic acid-binding region) (b), core protein (A1-D1-CB-6B1) against anti-(A1-D1 fraction) (c), keratan sulphate-rich region (A1-T-A1-CB-6B1) against anti-(A1-D1 fraction) (d), chondroitinase-digested chondroitin sulphate–peptides prepared from trypsin digests of proteoglycan aggregates (A1-T-A1-CB-6B2) against anti-(A1-T-A1 fraction) (e) and core protein against anti-(A1-T-A1 fraction) (f)*

Cross-reactivity between proteoglycan substructures

The specificities of the antibodies raised against the four preparations were further studied. Immunoelectromigration was used to establish the immunological identity between isolated hyaluronic acid-binding region and link-protein, isolated hyaluronic acid-binding region and keratan sulphate-rich region, link-protein and core protein, and finally keratan sulphate-rich region and peptides isolated from the chondroitin sulphate-rich region after trypsin digestion (A1-T-A1-CB-6B2; Fig. 4). None of the antigens tested cross-reacted, as shown by the crossing precipitates. The results show that the link-protein represents a protein not antigenically related to any structure in the proteoglycan monomer, in contrast with the previously proposed antigenic similarity (Keiser, 1975).

Corroborating data were obtained with peptide 'maps' of trypsin digests of reduced and alkylated isolated hyaluronic acid-binding region and link-protein (Fig. 5). The two proteins show non-identical patterns of peptides after digestion, indicating different primary structures.

Some preparations of antigens used for testing cross-reactivities were not rechromatographed before use. The precipitation patterns shown in Fig. 6 were obtained with such antigens on Ouchterlony double-diffusion against the antibodies used for testing. The isolated hyaluronic acid-binding region showed strong reactions of identity with anti-(A1-D1 fraction) and with anti-(hyaluronic acid-binding region). The anti-(link-protein) gave a faint reaction of non-identity with the hyaluronic acid-binding region

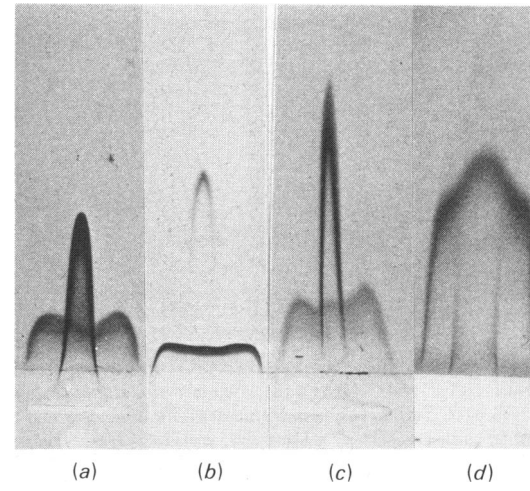


Fig. 4. *Immunoelectromigration showing the non-identity between (a) the link-protein (in the slit) and the hyaluronic acid-binding region (in the well) against a mixture of anti-(link-protein) and anti-(hyaluronic acid-binding region), (b) hyaluronic acid-binding region (in the slit) and keratan sulphate-rich region (A1-T-A1-CB-6B1) (in the well) against a mixture of anti-(A1-T-A1 fraction) and anti-(hyaluronic acid-binding region), (c) the link-protein (in the slit) and the core protein (in the well) against a mixture of anti-(A1-T-A1 fraction) and anti-(link-protein) and (d) the keratan sulphate-rich region (A1-T-A1-CB-6B1) (in the slit) and chondroitin sulphate peptides (A1-T-A1-CB-6B2) (in the well) against a mixture of anti-(A1-D1 fraction) and anti-(A1-T-A1 fraction)*

preparation used for testing, probably caused by a small contamination of link-protein that was absent from the preparation used for immunization. Corroborating this interpretation no reaction was observed between anti-(link-protein) and the purified hyaluronic acid-binding region preparation (results not shown). Conversely the link-protein preparation gave a strong reaction with the anti-(link-protein) and weak reactions with anti-(hyaluronic acid-binding region) and anti-(A1-D1 fraction). The two last structures showed identity, whereas the link-protein reacted with non-identity. The most likely explanation is that a contamination of hyaluronic acid-binding region is present in the link-protein preparation used for testing, but absent from the preparation used for immunization. Therefore it appears essential to rechromatograph samples to be used for immunization. The A1-D1 fractions reacted with anti-(A1-D1 fraction), with anti-(hyaluronic acid-binding region) and with anti-(A1-T-A1 fraction), as expected from the data discussed above. The isolated chondroitin sulphate–peptides (A1-T-A1-CB-6B2) reacted only with the anti-(A1-T-A1 fraction), with a

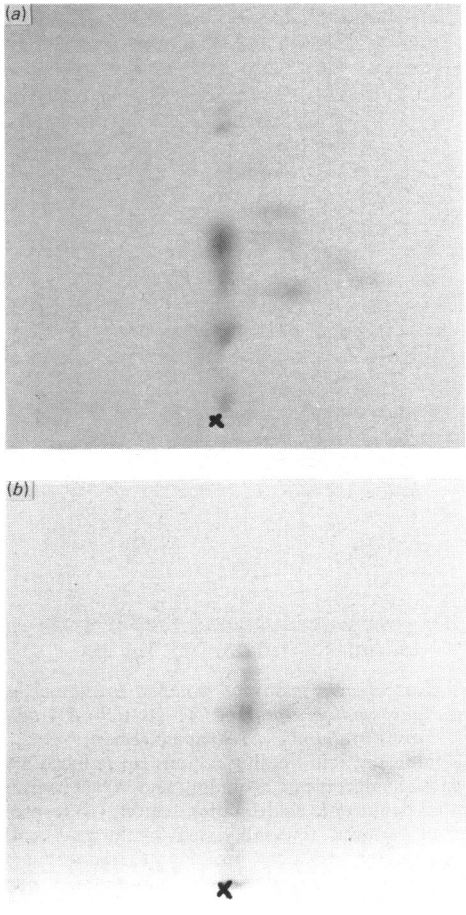


Fig. 5. *Peptide 'maps' of trypsin-digested reduced and alkylated hyaluronic acid-binding region (a) and link-protein (b)*

Electrophoresis was carried out (50V/cm for 30min) in the horizontal dimension and chromatography was carried out in the vertical dimension. The cross indicates the origin.

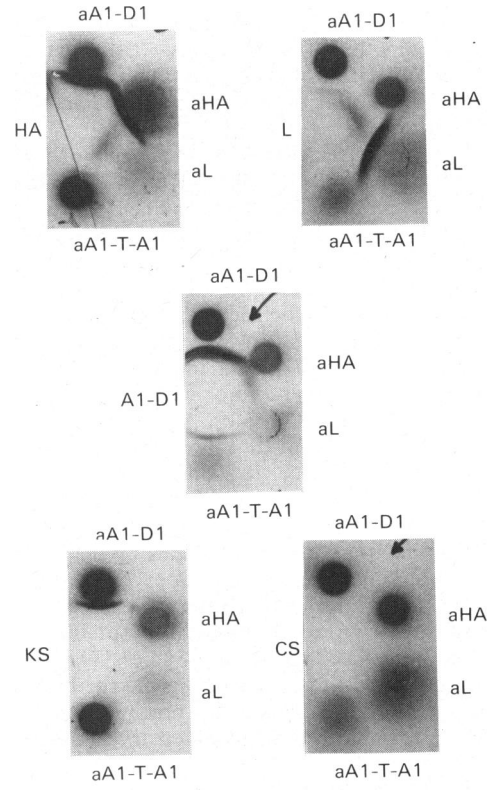


Fig. 6. *Ouchterlony double-diffusion analysis of antigen preparations against the different antisera*

Abbreviations: HA, hyaluronic acid-binding region; aHA, anti-(hyaluronic acid-binding region); A1-D1, A1-D1 fraction; aA1-D1, anti-(A1-D1 fraction); L, link-protein; aL, anti-(link-protein); A1-T-A1, A1-T-A1 fraction; aA1-T-A1, anti-(A1-T-A1 fraction); CS, chondroitin sulphate–peptides (A1-T-A1-CB-6B2); KS, keratan sulphate-rich region (A1-T-A1-CB-6B1).

precipitate close to the antibody well. The isolated keratan sulphate-rich region peptide, on the other hand, reacted only with the anti-(A1-D1 fraction), in accordance with data discussed above.

The reactivity of the antisera prepared can be summarized as follows: anti-(A1-D1 fraction) contains different antibodies reacting with the hyaluronic-acid-binding region, the keratan sulphate-rich region and possibly also antibodies directed against structures destroyed by the trypsin digestion used to cleave the molecule. The anti-(hyaluronic acid-binding region) and the anti-(link-protein) only contain antibodies specific for the hyaluronic acid-binding region and the link-protein respectively. The anti-(A1-T-A1 fraction) contains only detectable antibodies directed against the chondroitin sulphate–peptides present in the antigen.

Modification of proteoglycan structures and immunological reactivity

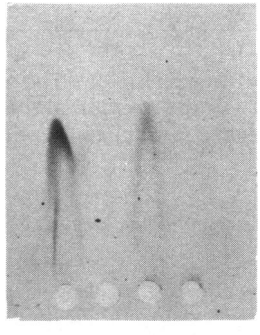
An attempt was made to design a procedure to obtain immunological reaction with selected structures of the proteoglycans by destruction of other potentially reactive structures. Could for example the antigenic reactivity of the hyaluronic acid-binding region with anti-(A1-D1 fraction) be destroyed without affecting or abolishing the reactivity of the keratan sulphate-rich region? Three types of modifi-

cations were tried: (1) direct trypsin digestion of antigens; (2) reduction of preparations with dithiothreitol and then alkylation with iodoacetamide; (3) reduction and alkylation of substances and then trypsin digestion.

The trypsin-digested link-protein did not form a precipitate with anti-(link-protein) (Fig. 7), indicating that the small peptides formed when the isolated protein was digested were non-antigenic. It should be noted that the link-protein preparation used both for immunization and testing was prepared from trypsin digests of aggregates. It is likely, then, that the link-protein in the aggregate was protected from fragmentation by trypsin by its interactions with the proteoglycan monomer and/or with hyaluronic acid (Heinegård & Hascall, 1974*a*). A weak reaction was obtained between anti-(link-protein) and trypsin-digested maleoylated or citraconoylated link-protein (results not shown). It is possible that the maleoylation or citraconylation of the lysine residues offered protection from attack at these sites by the enzyme. The larger fragments formed apparently retained some of the antigenicity. Reduced and alkylated maleoylated or citraconoylated link-protein reacted with the anti-(link-protein) antibodies. The size of the 'rocket' indicated that no antigenic site had been destroyed by the reduction. No precipitate was obtained with reduced and alkylated trypsin-digested link-protein.

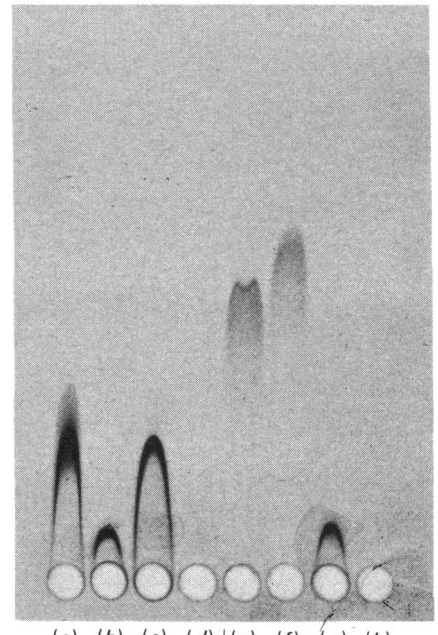
The reduced and alkylated or the trypsin-digested hyaluronic acid-binding-region peptide reacted and each gave one precipitate both when tested against anti-(hyaluronic acid-binding region) and against anti-(A1-D1 fraction) (Figs. 8*a*–8*c* and 9*a*–9*c*). The 'rockets' were lower than those of the intact antigen, indicating that some sites had been destroyed by the action of the enzyme or by the reduction and alkylation procedure, probably owing to unfolding of the protein. In a separate experiment, however, it was shown that the intact and the trypsin-digested hyaluronic acid-binding region showed a major reaction of identity on immunoelectromigration (Fig. 10*b*), indicating that few antigenic determinants had been destroyed by the action of the enzyme. In contrast, when the hyaluronic acid-binding region had been reduced and alkylated and subsequently digested with trypsin no precipitate was obtained with the antibodies tested (Figs. 8*d* and 9*d*). Therefore it appears that degradation of the hyaluronic acid-binding region by trypsin is more extensive when the protein had first been unfolded by the reduction procedure.

Corroborating results were obtained when the core protein was similarly treated with trypsin with or without previous reduction and alkylation as discussed above. Reduction and alkylation did not alter the precipitation pattern significantly when com-



(*a*) | (*b*) | (*c*) | (*d*)

Fig. 7. *Electroimmunoassay of maleoylated link-protein preparations against anti-(link-protein)*
(*a*) Link-protein; (*b*) trypsin-digested link-protein; (*c*) reduced and alkylated link-protein; (*d*) trypsin-digested reduced and alkylated link-protein.

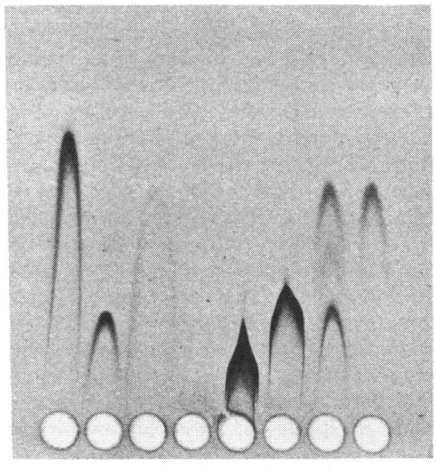


(*a*) (*b*) (*c*) (*d*) | (*e*) (*f*) (*g*) (*h*)

Fig. 8. *Electroimmunoassay of modified hyaluronic acid-binding region and core protein (A1-D1-CB-6B1) against anti-(hyaluronic acid-binding region)*
(*a*) Hyaluronic acid-binding region; (*b*) reduced and alkylated hyaluronic acid-binding region; (*c*) trypsin-digested hyaluronic acid-binding region; (*d*) trypsin-digested reduced and alkylated hyaluronic acid-binding region; (*e*) core protein; (*f*) reduced and alkylated core protein; (*g*) trypsin-digested core protein; (*h*) trypsin-digested reduced and alkylated core protein.

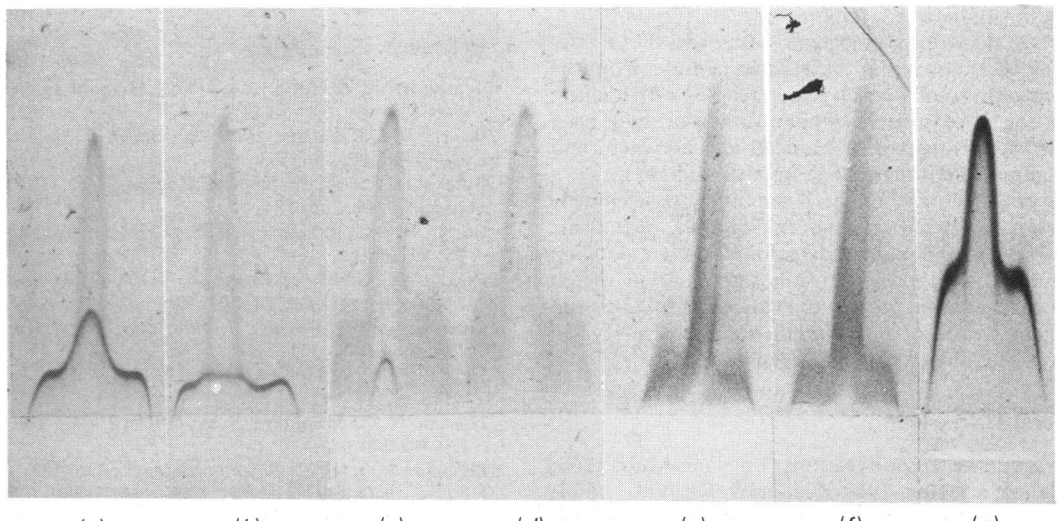
pared with the intact material (Figs. 8*f* and 9*f*), indicating that the antigenicity was essentially intact. In contrast digestion of the core protein with trypsin yielded a preparation that gave three precipitate 'rockets' against anti-(A1-D1 fraction) and only one against anti-(hyaluronic acid-binding region) (Figs. 8*g* and 9*g*). When the core protein was reduced and alkylated and then digested with trypsin, only one 'rocket' of the same appearance as one of the 'rockets' obtained with the direct trypsin digest of the core protein was observed with anti-(A1-D1 fraction) (Fig. 9*h*). As expected, the anti-(hyaluronic acid-binding region) did not react with the reduced, alkylated and trypsin-treated core protein (Fig. 8*h*). It appears, then, that the antigenic reactivity of the hyaluronic acid-binding region was not destroyed by the action of trypsin alone. Reduction and alkylation, and then trypsin digestion, destroyed the antigenic sites of the hyaluronic acid-binding region, whereas one antigenic site of the core protein still reacted. On the basis of the data discussed above it is likely that this site represents the keratan sulphate-rich region.

Further information was obtained when immunoelectromigration was used to test the cross-reactivity of the hyaluronic acid-binding region, keratan sulphate-rich region and chondroitin sulphate–peptides (A1-T-A1-CB-6B2) with the core protein that had been digested with trypsin before or after alkylation (Fig. 10). When electrophoresed against



(*a*) (*b*) (*c*) (*d*) (*e*) (*f*) (*g*) (*h*)

Fig. 9. *Electroimmunoassay of modified hyaluronic acid-binding region and core protein against anti-(A1-D1 fraction)*
(*a*)–(*h*) were the same antigens as in Fig. 8.



(*a*) (*b*) (*c*) (*d*) (*e*) (*f*) (*g*)

Fig. 10. *Immunoelectromigration analysis of* (*a*) *hyaluronic acid-binding region* (*in the slit*) *and trypsin-digested core protein* (*in the well*) *against anti-*(*A1-D1 fraction*), (*b*) *hyaluronic acid-binding region* (*in the slit*) *and trypsin-digested reduced and alkylated core protein* (*in the well*) *against anti-*(*A1-D1 fraction*), (*c*) *keratan sulphate-rich region* (*A1-T-A1-CB-6B1*) (*in the slit*) *and trypsin-digested core protein* (*in the well*) *against anti-*(*A1-D1 fraction*), (*d*) *keratan sulphate-rich region* (*in the slit*) *and trypsin-treated reduced and alkylated core protein* (*in the well*) *against anti-*(*A1-D1 fraction*), (*e*) *chondroitin-sulphate–peptides* (*A1-T-A1-CB-6B2*) (*in the slit*) *and trypsin-digested core protein* (*in the well*) *against anti-*(*A1-T-A1 fraction*), (*f*) *chondroitin sulphate–peptides* (*A1-T-A1-CB-6B2*) (*in the slit*) *and trypsin-treated reduced and alkylated core protein* (*in the well*) *against anti-*(*A1-T-A1 fraction*) *and* (*g*) *trypsin-digested hyaluronic acid-binding region* (*in the slit*) *and hyaluronic acid-binding region* (*in the well*) *against anti-*(*hyaluronic acid-binding region*)

anti-(A1-D1 fraction) the trypsin-digested core protein contained one component that showed reaction of identity with the isolated hyaluronic acid-binding region and in addition one component that gave a higher 'rocket' not extending to the starting point (Fig. 10*a*). The latter component probably represents the keratan sulphate-rich region. When the trypsin-digested reduced and alkylated core protein was electrophoresed only one component (the high 'rocket') was observed showing reaction of non-identity with the isolated hyaluronic acid-binding region (Fig. 10*b*). Therefore in support of the data discussed above it appears that reduction and alkylation followed by trypsin digestion of the core protein destroys the immunological reactivity of the hyaluronic acid-binding region, whereas the reactivity of another fragment remains. Further information was obtained when the cross-reactivity with the isolated keratan sulphate-rich region was tested with anti-(A1-D1 fraction). It was shown that the component (giving a high 'rocket') in the proteoglycan monomer that was resistant to reduction and alkylation followed by trypsin digestion reacted with partial identity with the keratan sulphate-rich region (Figs. 10*c*, 10*d* and 9*g*). Therefore it appears that there is a third site in trypsin-digested proteoglycan monomer core preparations that reacts with the anti-(A1-D1 fraction) and is identical neither to the isolated hyaluronic acid-binding region nor to the keratan sulphate-rich region.

When electrophoresed against anti-(A1-T-A1 fraction) it was shown that the isolated peptides from the chondroitin sulphate-rich region reacted with identity with the core protein preparation that had been either trypsin-treated or reduced and alkylated and then digested with trypsin (Figs. 10*e* and 10*f*).

In conclusion, then, it is possible to selectively identify and determine the link-protein, hyaluronic acid-binding region, keratan sulphate-rich region and peptides from the chondroitin sulphate-rich region by using a combination of the various antibodies and antigens before or after fragmentation with trypsin of reduced and alkylated antigens.

General Discussion

In a number of publications (Loewi & Muir, 1965; Tsiganos & Muir, 1969; Keiser & De Vito, 1974; Keiser, 1975) antibodies have been raised against proteoglycan preparations of various purities. Usually several precipitate lines have been obtained in Ouchterlony double diffusion, indicating either that the preparations contained impurities and/or that they contained several antigenic sites. More recently the link-protein and the substructures of the proteoglycan protein core have been discovered (Hascall & Sajdera, 1969; Keiser *et al.*, 1972; Heinegård & Hascall, 1974*a*; Heinegård & Axelsson, 1977). Keiser and his co-worker (Keiser & De Vito, 1974; Keiser, 1975) arrived at the conclusion that link-protein and proteoglycan core protein were antigenically related. The results reported in the present paper show that this is not the case and also indicate that the primary structures of the hyaluronic acid-binding region and the link-protein differ. It is likely that the results obtained by Keiser and his co-worker (Keiser & De Vito, 1974; Keiser, 1975) were due to use of impure antigens (compare Fig. 6 in the present paper). In the present work it is shown that antibodies specifically reacting with link-protein, hyaluronic acid-binding region, keratan sulphate-rich region and chondroitin sulphate–peptides from the chondroitin sulphate-rich region respectively can be raised in rabbits. Thus the proteoglycan monomer contains at least three different antigenic sites.

The use of antibodies, in combination with fragmentation of the antigens as discussed above, should be very useful in studies of proteoglycan biosynthesis and in studies of variations of proteoglycan structure in normal and degenerated tissues.

This work was supported by the Swedish Medical Research Council (13X-00139-13B), Konung Gustaf V:s 80-års fond, Koch's stiftelse, Segerfalk's stiftelse and the Medical Faculty, University of Lund. J. W. is a recipient of a Graduate Training Grant from the Medical Faculty, University of Lund. Skilful technical assistance by Rosmari Sandfalk is greatly appreciated.

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