Immunodiffusion studies of the tryptic fragments of bovine nasal-cartilage proteoglycan monomer of high buoyant density

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Tryptic fragments of bovine nasal-cartilage proteoglycan, fractionated by dissociative density-gradient ultracentrifugation, were made to react by immunodiffusion against antiserum to a hyaluronidase-digest subfraction of cartilage proteoglycan monomer. This reaction produced two families of partly superimposed precipitin lines. One family was restricted to gradient fractions of medium or low buoyant density and included the immunoprecipitation reaction attributed to the hyaluronic acid-binding region of the cartilage proteoglycan monomer. The second family of precipitin lines was present alone in gradient fractions of high buoyant density. Immunodiffusion studies with antisera to relatively homogeneous keratan sulphate-rich and chondroitin sulphate-bearing fragment subfractions isolated from the gradient fraction of highest density indicated that both subfractions contained the antigenic determinants responsible for the second family of precipitin lines. Additional immunodiffusion studies, with the use of multispecific antisera to chondroitinase ABC digest and hyaluronidase digest of proteoglycan monomer, confirmed that the two subfractions shared antigenic determinants, and, in addition, indicated that these determinants were on one molecular species in the keratan sulphate-rich fragment subfraction and divided among at least three in the chondroitin sulphate-bearing fragment subfraction. Although an unprecedentedly large number of cartilage proteoglycan antigens could be recognized with the antisera employed in this study, it was not possible to identify antigenic determinants unambiguously specific for the three structurally and functionally distinct regions of the cartilage proteoglycan monomer.

The cartilage proteoglycan monomer is thought to consist of three chemically and functionally distinct regions: a glycoprotein region responsible for aggregation with hyaluronic acid at one end of the linear protein core, a region of variable lengths of protein core bearing chondroitin sulphate chains at the other end, and a region of protein core with keratan sulphate side chains in between (reviewed by Rosenberg, 1975; Hascall, 1977). The immunogenicity of cartilage proteoglycan monomer is due solely to its core protein (Keiser & DeVito, 1974; Sparks *et al.*, 1980), and each of the distinct regions of the monomer is believed to have immunological specificity. We initially described a 'CS' antigenic determinant based on the immunodiffusion reactivity of chondroitin sulphate-containing peptides (Keiser & DeVito, 1974). This was distinguished from an antigenic determinant termed 'KS' associated with proteoglycan monomer fragments complexed with hyaluronic acid (Keiser, 1975). Wieslan-

der & Heinegård (1979) subsequently identified an antigen associated with the keratan sulphate-rich region of the proteoglycan monomer and developed specific electroimmunoassay and radioimmunoassay procedures for each of the three regions of the proteoglycan monomer (Wieslander & Heinegård, 1980).

In the preceding paper (Keiser *et al.*, 1982), we described a procedure for the fractionation of the tryptic fragments of bovine nasal-cartilage proteoglycan by equilibrium density-gradient centrifugation under dissociative conditions, and for the isolation from the fractions of highest buoyant density of relatively homogeneous fragment subfractions corresponding to the keratan sulphate-rich and chondroitin sulphate-bearing regions of the proteoglycan monomer. In the present paper I describe immunodiffusion studies with these tryptic fragment fractions of cartilage proteoglycan monomer, the results of which are not in accord with the

specific correspondence of three sets of antigenic determinants with the three regions of the proteoglycan monomer core protein.

Materials and methods

Proteoglycan fractions

The details of the extraction of bovine nasal cartilage proteoglycan, trypsin digestion, purification by ion-exchange chromatography, fractionation by equilibrium density-gradient centrifugation and the preparation and characterization of subfractions B/6B-2 and B/6B-4 are presented in the preceding paper (Keiser *et al.*, 1982). A preparation of fraction B/6B-2 (5 mg/ml in 0.1M-Tris/acetate buffer, pH 7.4) was digested with α -chymotrypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) (25 μ g/ml) at 37°C for 4 h. Proteoglycan monomer was prepared by the standard associative and dissociative density-gradient centrifugation method of Hascall & Sajdera (1969), as described previously (Keiser *et al.*, 1972). The trypsin + chymotrypsin digest fraction defining the 'CS' antigenic determinant was the same as that used in previous studies (Keiser & DeVito, 1974). Chondroitin sulphate was prepared from proteoglycan monomer by β -elimination (5 mg/ml in 0.4M-NaOH for 18 h) and elution from DEAE-cellulose between 0.5M- and 1M-KCl in 10mM-sodium phosphate buffer, pH 7. Chondroitin sulphate (5 mg/ml) was digested with chondroitinase ABC (0.025 nominal unit/ml) for 18 h at 37°C under toluene.

Antisera

Rabbits were immunized with 1 mg of fraction B/6B-2 or fraction B/6B-4 in 0.9% NaCl homogenized with an equal volume of Freund's complete adjuvant injected intramuscularly and in several subcutaneous sites. At the same time, the rabbits were also given an intramuscular injection of 0.25 ml of pertussis vaccine. Booster injections of 0.5 mg of immunizing antigen in incomplete Freund's adjuvant were given at 4-week intervals, with serum being obtained 7–10 days afterwards. Sera were preserved with phenol and stored at 4°C. The sera denoted as antiserum BN42B and antiserum BN43B were taken from the second bleeding. Antisera 40A and 41B were prepared in a similar manner, with material from the included Sepharose 4B peak of chondroitinase ABC digest of proteoglycan monomer (Keiser & Hatcher, 1977) as immunogen for antiserum BN40A, and material from pool II of the Sepharose 6B eluate of proteoglycan monomer digested with Leo hyaluronidase (Keiser & Hatcher, 1977) as immunogen for antiserum BN41B. Before use, antisera were concentrated three times by vacuum ultrafiltration and absorbed with 20 mg of freeze-dried dialysed bovine serum/ml of concen-

trated antiserum. Portions of several antisera were absorbed with freeze-dried chondroitinase ABC digest of isolated chondroitin sulphate (30 mg/ml of concentrated serum) or with fraction B/6B-2 or B/6B-4 (5 mg/ml of concentrated serum).

Immunodiffusion

Immunodiffusion was performed in 0.6% agarose at pH 8.6 by standard methods (Ouchterlony, 1962). Proteoglycan antigen concentrations were 2–5 mg/ml for chondroitinase-digested fractions and 10–15 mg/ml for fractions not treated with chondroitinase. Chondroitinase-digested chondroitin sulphate was used at a concentration of 15 mg/ml. Plates were incubated for 48–72 h at 4°C, washed with several changes of 0.9% NaCl and stained with Coomassie Blue.

Results

Density-gradient fractions

The dissociative density-gradient subfractions of the glycosaminoglycan-bearing tryptic fragments of bovine nasal-cartilage proteoglycan, the preparation of which was described in the preceding paper (Keiser *et al.*, 1982), were made to react by immunodiffusion against antiserum BN41B, a multivalent antiserum to bovine cartilage proteoglycan monomer unreactive with link proteins. The resultant set of precipitin reactions demonstrated two families of immunoreactive fragments, as shown in Fig. 1. The first family consisted of one or perhaps two precipitin lines immediately adjacent to the antibody well. This reaction was present in all density-gradient fractions, although it was often indistinct with the fractions of greatest density, e.g. fractions 1 and 2 in Fig. 1. The second family of immunoprecipitins, located nearer to the antigen wells, was found only in fractions of medium and low density. It consisted of at least four precipitin lines whose relative intensity and distinctness from each other varied from fraction to fraction in an inconsistent manner.

Antisera to fractions B/6B-2 and B/6B-4

The nature of the immunoprecipitation reaction nearer to the antibody well in Fig. 1 was studied by using antisera to the keratan sulphate-rich fragment fraction B/6B-2 and the chondroitin sulphate-bearing fragment fraction B/6B-4 prepared from the CsCl-density-gradient fraction of highest density as described in the preceding paper (Keiser *et al.*, 1982). The antisera to fractions B/6B-2 and B/6B-4, designated 'BN42B' and 'BN43B' respectively, each formed single precipitin lines on immunodiffusion against both fractions B/6B-2 and B/6B-4, and when the two fractions were placed in adjacent wells these precipitin lines merged smoothly in a reaction of

Fig. 1. Immunodiffusion of glycosaminoglycan-associated tryptic fragments of bovine nasal-cartilage proteoglycan fractionated by dissociative equilibrium density-gradient centrifugation against an antiserum to proteoglycan monomer protein core produced by testicular hyaluronidase (BN41B)
For full experimental details see the text.

identity (Fig. 2a, left side of schematic and photograph). Similarly, when the two antisera were placed in adjacent wells, the precipitin line formed by each diffusing against fraction B/6B-2 or fraction B/6B-4 also appeared to merge smoothly in a reaction of identity (Fig. 2b). Absorption of antiserum BN42B with fraction B/6B-4 (5 mg/ml), or of antiserum BN43B with fraction B/6B-2 (5 mg/ml), resulted in the loss of the precipitin line formed by the immunodiffusion reaction of these antisera with fractions B/6B-2 or B/6B-4 respectively (results not shown).

Antisera to proteoglycan monomer

Additional studies of the immunological properties of the proteoglycan fragments of high buoyant density were performed with antisera BN40A and BN41B raised against chondroitinase ABC and testicular, hyaluronidase digests of proteoglycan monomer respectively. A hypothesis that accounts for the immunodiffusion findings is presented schematically in Fig. 3.

Antiserum BN40A, like antisera BN42B and BN43B, produced smoothly merging single precipitin lines on immunodiffusion against fractions B/6B-2 and B/6B-4 in adjacent wells (Fig. 2a, right), and the precipitin lines formed by antisera BN42B or BN43B and antiserum BN40A in adjacent wells reacting against fraction B/6B-2 also merged smoothly (Fig. 2c, left). However, the precipitin lines formed by these antisera on reaction with fraction B/6B-4 diverged widely (Fig. 2c, right). This finding indicates that antisera BN42B and BN43B on the one hand and antiserum BN40A on the other recognize different sets of antigenic determinants (----- and ———— respectively in Fig. 3), and that these antigenic determinants are located on separate

core protein fragments in fraction B/6B-4, but not in fraction B/6B-2 (Fig. 3).

Antiserum BN41B also produced a single precipitin line on reaction with fraction B/6B-2 that merged smoothly with the precipitin line produced by the reaction of antisera BN40A, BN42B or BN43B in an adjacent well (Figs. 4a, left, and 4b, left). In contrast with the other antisera, antiserum BN41B produced two precipitin lines on immunodiffusion against fraction B/6B-4 (Figs. 4a, right, and 4c, left; it is indistinct in Fig. 4b, right). These two precipitin lines appeared to merge into the single precipitin line produced by the reaction of antiserum BN41B with fraction B/6B-2 (Fig. 4c, left), and neither merged with the precipitin line formed by the reaction of antiserum BN40A with fraction B/6B-4 (Fig. 4b, right). These observations indicate that antiserum BN41B recognizes two sets of antigenic determinants (depicted as ○○○○○ and ××××× in Fig. 3), which are different from the set of determinants recognized by antiserum BN40A (——— in Fig. 3), and that there are at least three sets of antigenic determinants present on separate molecular species in fraction B/6B-4 that appear to be on the same molecule in fraction B/6B-2 (Fig. 3).

Relationship to the 'CS' antigenic determinant

Antisera BN42B, BN43B and BN40A did not form a precipitin line on reaction with the trypsin + chymotrypsin-digest subfraction that defines the 'CS' antigenic determinant (Keiser & DeVito, 1974) (Figs. 4a, right, and 4c, right). Antiserum BN41B did react with the 'CS' antigenic determinant (○○○○○ in Fig. 3), and on immunodiffusion against the appropriate fractions diffusing from adjacent wells it was found that the 'CS' precipitin line merged smoothly with the single precipitin line formed by the reaction of antiserum BN41B with

Fig. 2. Immunodiffusion of keratan sulphate-rich fragments (fraction B/6B-2) and chondroitin sulphate-bearing fragments (fraction B/6B-4) from bovine nasal-cartilage proteoglycan against antisera to fraction B/6B-2 (BN42B), fraction B/6B-4 (BN43B) and proteoglycan monomer protein core produced by chondroitinase ABC (BN40A) For full experimental details see the text.

Reactivity of antisera		Antigenic composition of proteoglycan fractions	
Antisera	Antigenic determinants recognized	Fraction	Antigenic fragments
BN42B	xxxxx	B/6B-2	ooooo-----xxxxx
BN43B	-----	B/6B-4	ooooo----- -----xxxxx
BN40A	-----	B/6B-2/chy	ooooo----- xxxxx
BN41B	ooooo ('CS') xxxxx		

Fig. 3. Schematic hypothesis explaining the immunodiffusion results described in the present paper in terms of the antigenic determinants recognized by individual antisera and the antigenic composition of the high-buoyant-density tryptic fragments of cartilage proteoglycan The derivations of the antisera are indicated in the captions to Figs. 1 and 2. B/6B-2/chy represents the chymotrypsin digest of fraction B/6B-2.

fraction B/6B-2 and also with the precipitin line nearer to the antibody well formed by the reaction of antiserum BN41B with fraction B/6B-4 (Fig. 4c, left). Although antiserum BN43B did not form a

precipitin reaction with the 'CS' antigenic determinant, both the 'CS'-related and the 'CS'-unrelated lines formed by the reaction of antiserum BN41B and fraction B/6B-4 merged with the single pre-

Fig. 4. Immunodiffusion of keratan sulphate-rich fragments (fraction B/6B-2), chondroitin sulphate-bearing fragments (fraction B/6B-4) and fragments bearing the 'CS' antigenic determinant against antisera to proteoglycan fractions. The origin of the antisera used is indicated in the captions for Figs. 1 and 2. For full experimental details see the text.

cipitin line formed by the reaction of antiserum BN43B in an adjacent well with fraction B/6B-4 (Fig. 4a, right). This suggests that antiserum BN43B recognizes an antigenic determinant in both fraction B/6B-2 and fraction B/6B-4 (shown as ----- in Fig. 3) that is different from those recognized by antiserum BN41B, and that this antigenic determinant is present on at least two fragments in fraction B/6B-4, one fragment bearing in addition the 'CS' determinant (OOOOO-----) and the second bearing also the non-'CS' determinant recognized by antiserum BN41B (-----x x x x x) (Fig. 3).

Chymotrypsin digestion of fraction B/6B-2

Fraction B/6B-2 was digested with chymotrypsin and the digest was made to react by immunodiffusion against the four anti-proteoglycan sera. The reaction of chymotrypsin-treated fraction B/6B-2 and antiserum BN41B formed two precipitin lines, which tended to be more widely separated than the two precipitin lines formed by the reaction of

antiserum BN41B with fraction B/6B-4 in an adjacent well, but merged smoothly with them (Fig. 5a, left). On immunodiffusion against chymotrypsin-digested fraction B/6B-2, antisera BN42B and BN43B also formed two precipitin lines, which merged smoothly into the single line formed by the reaction of the antiserum with fraction B/6B-4 in an adjacent well (Fig. 5b). Each of the two precipitin lines formed by the immunodiffusion reaction of chymotrypsin-digested B/6B-2 with antiserum BN42B or BN43B appeared to merge smoothly with the corresponding precipitin line formed by the reaction with antiserum BN41B in an adjacent well (results not shown). The immunodiffusion reaction of antiserum BN40A with fraction B/6B-2 was unchanged by chymotrypsin digestion (Fig. 5a, right), and the precipitin line formed by the reaction of antiserum BN40A and the chymotrypsin digest of fraction B/6B-2 diverged from those formed by the reactions of antisera BN41B and BN43B with the chymotryptic digest (results not shown). Chymo-

Fig. 5. Immunodiffusion of the chymotrypsin digest of keratan sulphate-rich fragments (fraction B/6B-2/C), chondroitin sulphate-bearing fragments (fraction B/6B-4) and fragments bearing the 'CS' antigenic determinant against antisera to proteoglycan fractions

The origin of the antisera used is indicated in the captions for Figs. 1 and 2. For full experimental details see the text. In the immunodiffusion plate shown here, the fraction B/6B-2 used for chymotrypsin digestion was prepared from Sepharose 4B pool 3 (Fig. 2 in Keiser *et al.*, 1982) rather than from Sepharose 4B pool 1. The precipitin reactions near the antigen wells, shown as stipples in the schematics, were not present when the chymotrypsin digest of fraction B/6B-2 from Sepharose 4B pool 1 was used and are due to contamination by proteoglycan fragments of lower buoyant density.

Fig. 6. Immunodiffusion studies comparing the reactivity of tryptic fragments of cartilage proteoglycan of medium and high buoyant density (fractions M/6B-2 and B/6B-4 respectively)

The origin of the antisera used is shown in the captions for Figs. 1 and 2. For full experimental details see the text.

trypsin digestion of fraction B/6B-2 thus resulted in separation of its antigenic determinants into several independently diffusing molecular species similar to the fragments in fraction B/6B-4 (Fig. 3).

Relation to other proteoglycan antigens

The distinctiveness of the antigenic determinants associated with proteoglycan fragments of high

buoyant density in fractions B/6B-2 and B/6B-4 from those restricted to fragments of medium and low buoyant density was confirmed by finding that antisera BN42B and BN43B, raised against fraction B/6B-2 and fraction B/6B-4 respectively, reacted with the same antigenic determinants on immunodiffusion against fractions of high-density and medium-density fragments (Fig. 6a, left), under conditions where antisera BN40A and BN41B, raised against proteoglycan monomer protein core, formed an additional set of precipitin lines on immunodiffusion against medium-density fragments (Figs. 6a, right, and 6b). The bowing of one of the precipitin lines due to mid-gradient antigens formed by reaction with antiserum BN40A as it approached the well containing fraction B/6B-4 (Fig. 6b, left) suggests that fraction B/6B-4 is contaminated by small amounts of a mid-gradient antigen that is probably also responsible for the fuzzy precipitin reactions near the well containing fraction B/6B-4 in Figs. 2a (right), 2c (right), 4c (right) and 5a (right), shown as stipples in the accompanying schematics. (See also the caption to Fig. 5.)

Although antisera BN40A, BN42B and BN43B were prepared with chondroitinase-digested proteoglycan fractions as immunogens, they did not form a precipitin line on immunodiffusion against a chondroitinase ABC digest of chondroitin sulphate, and absorption of these antisera with the chondroitinase digest of chondroitin sulphate did not affect their immunodiffusion reactions with proteoglycan fractions (results not shown). Furthermore, the precipitin lines formed on the immunodiffusion of these antisera against proteoglycan fractions digested with trypsin and chondroitinase ABC formed reactions of identity with the precipitin lines formed on the immunodiffusion of these antisera against proteoglycan fractions digested with trypsin only (results not shown).

Discussion

Several firm conclusions can be drawn from the immunodiffusion results given in the present paper, as listed below.

(1) Digestion of proteoglycan aggregate with trypsin produces at least eight chemically or immunologically distinct monomer fragments: at least one in fraction B/6B-2, three in fraction B/6B-4 and four more in fragment fractions of medium and low buoyant density.

(2) On the basis of previously reported immunodiffusion findings with antisera similar to antiserum BN41B in origin and reactivity (Keiser, 1975), it is clear that one or more of the precipitin lines restricted to fragment fractions of medium and low buoyant density is due to the reaction of antiserum

BN41B with the hyaluronic acid-binding portion of the cartilage proteoglycan monomer.

(3) Although the subfractions of high-buoyant-density tryptic fragments from the keratan sulphate-rich and chondroitin sulphate-bearing regions of the cartilage proteoglycan monomer, fractions B/6B-2 and B/6B-4, are chemically quite different (Keiser *et al.*, 1982), they share the same antigenic determinants. This may in part be related to the presence of a chondroitin sulphate-bearing segment in the keratan sulphate-rich fragment in fraction B/6B-2 (Keiser *et al.*, 1982).

(4) The same antigenic determinants are associated with physically different forms in fractions B/6B-2 and B/6B-4. The simplest interpretation of the immunodiffusion findings is that the antigenic determinants present on a single tryptic fragment in fraction B/6B-2 are distributed among three fragments in fraction B/6B-4, and that chymotrypsin digestion of fraction B/6B-2 yields subfragments similar to, although not necessarily identical with, those in fraction B/6B-4. The clear-cut differences noted in the immunodiffusion reactivity of fractions B/6B-2 and B/6B-4 serve to rule out cross-contamination as the reason for the apparent antigenic overlap. Similarly, the chemical differences between the two fractions (Keiser *et al.*, 1982) make it highly unlikely that all the fragments in fraction B/6B-4 could have arisen simply from partial proteolysis of fraction B/6B-2 by contaminant proteinases during extraction and fractionation. Furthermore, there is no necessary contradiction between the conclusion that fraction B/6B-2 contains a single molecular species and fraction B/6B-4 has three, and the finding on end-group analysis that each fraction has two *N*-terminal amino acids (Keiser *et al.*, 1982). The two polypeptide chains in fraction B/6B-2 may be covalently linked or non-covalently bound yet non-dissociable in 4M-guanidinium chloride, one polypeptide may not be immunoprecipitated, or the immunodiffusion reaction of fraction B/6B-2 may actually consist of two superimposed precipitin lines. Fraction B/6B-4 may have two polypeptide chains with the same *N*-terminal amino acid, or a polypeptide chain with an *N*-terminal amino acid not identifiable by the method used.

(5) Although three of the four antisera used in this study were raised against chondroitinase-digested antigens, antibodies directed against the unsaturated uronic acid residues produced by the enzyme (Christner *et al.*, 1980), even if present, could not be responsible for the immunodiffusion findings.

Close inspection of the photographs and schematics provided in the present paper will reveal a number of inconsistencies (e.g. a precipitin line near the well containing antiserum BN43B on reaction with fraction B/6B-4 in Fig. 4a but not Fig. 5b), ambiguities [e.g. is the major precipitin line

formed by the reaction of antiserum BN43B and fraction B/6B-4 partially split (Fig. 4a, right) or merely broad with a single tail (Figs. 2a, 2b and 2c?) and discrepancies (e.g. the absence of the expected precipitin spur in the reaction of antiserum BN41B with fraction B/6B-2 and 'CS' fragments in Fig. 4c, left). These difficulties point to the complexity of the system under study, and, although they may affect some of the details of interpretation illustrated schematically in Fig. 3, they do not affect the conclusions outlined above.

The immunodiffusion findings described in the present paper differ from previously reported studies (Keiser & DeVito, 1974; Wieslander & Heinegård, 1979, 1980) with respect to the number and the specificity of the immunoreactive tryptic fragments of cartilage proteoglycan monomer. The discrepancies between the present findings and those obtained by Wieslander & Heinegård (1979, 1980) are probably due to differences in the proteoglycan fractions studied resulting from the different methods employed in their preparation. Thus their hyaluronic acid-binding fragment fraction was isolated as part of a hyaluronic acid-link protein-monomer fragment complex (Heinegård & Hascall, 1974), whereas the gradient fractions of medium and low buoyant density used in the present study could contain glycosaminoglycan-poor fragments from previously undefined regions of the proteoglycan monomer or from different proteoglycan monomer species (Roughley & Mason, 1976; Stanescu *et al.*, 1977; Swann *et al.*, 1979; Heinegård & Hascall, 1979; Heinegård *et al.*, 1981) in addition to the hyaluronic acid-binding fragment studied by Wieslander & Heinegård (1979, 1980). Immunological differences between the keratan sulphate-rich and chondroitin sulphate-bearing fragment fractions used in the present study, which share antigenic determinants, and the corresponding fractions used by Wieslander & Heinegård (1979, 1980), which are antigenically distinct, may reflect the biochemical differences noted in the preceding paper (Keiser *et al.*, 1982).

The existence of unresolved immunological discrepancies related to proteoglycan structure raises the potential for ambiguity in the immunological identification of proteoglycan components. Antisera to cartilage proteoglycan, even if monospecific, may detect only one of several hyaluronic acid-binding regions or chemically similar proteoglycan components, or they may not be able to distinguish between

chondroitin sulphate-bearing and keratan sulphate-rich monomer regions. Studies that attempt to identify the distinct structural and functional portions of cartilage proteoglycan monomer in tissues or tissue extracts by using techniques such as immunofluorescence or immune binding assays, which are critically dependent on the specificity of the antiserum employed, must therefore be interpreted with caution.

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