Immunological Studies of Bovine Nasal Cartilage Proteoglycan "Link Proteins" †

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ABSTRACT: Bovine nasal cartilage proteoglycan aggregates are dissociated and separated by density gradient centrifugation in 4 *M* guanidine into proteoglycan subunit (PGS) and glycoprotein link (GPL) fractions, the latter containing hyaluronic acid and "link proteins" responsible for aggregate formation. It was previously concluded on the basis of immunodiffusion studies that GPL has two antigenic components, one in common with PGS and one specific for the link proteins. However, in the present study it was found that antisera to PGS, which should lack link proteins, reacted with both "subunit" and "link" components of GPL, and antisera to fragments of PGS derived from the hyaluronic acid-binding portion of the molecule reacted preferentially with the link component. Reduction and alkylation of GPL led to modification of the reactions of both anti-GPL and anti-PGS sera with its link component. These immunodiffusion results indicate that the proteoglycan subunit and the link proteins are immunologically related and suggest that the link proteins may be identical with and derived from the hyaluronic acid binding portion of the proteoglycan subunit.

Bovine nasal cartilage proteoglycan consists of aggregates which can be separated into two distinct fractions by CsCl density gradient centrifugation in 4 *M* guanidine (Sajdera and Hascall, 1969; Hascall and Sajdera, 1969). The proteoglycan subunit fraction (PGS),[1] at the bottom of the gradient, contains chondroitin sulfate and keratan sulfate chains attached covalently to a core protein. Mixture of this fraction with small amounts of the glycoprotein link fraction (GPL), from the top of the gradient, results in the reaggregation of proteoglycan subunits as shown by an increase in viscosity and apparent molecular size and by the appearance of a more rapidly sedimenting peak on analytic ultracentrifugation. The major aggregation factor in GPL has been shown to be hyaluronic acid (Hardingham and Muir, 1972, 1974; Hascall and Heinegård, 1974a); however, two link proteins, demonstrable by sodium dodecyl sulfate gel electrophoresis of GPL (Keiser et al., 1972), also appear to play a role in aggregate formation (Gregory, 1973; Hascall and Heinegård, 1974a,b).

In our initial immunological study of the proteoglycan fractions produced by density gradient centrifugation, we found that PGS contained a single antigenic component, whereas proteoglycan aggregates and GPL contained two antigenic components, one related to PGS and a second which appeared to be associated with the link proteins of GPL (Keiser et al., 1972). These findings were confirmed by DiFerrante et al. (1972) who stressed the sequestered character of the link-associated antigen.

Subsequent studies have focused on the structure and antigenic composition of the proteoglycan subunit. As illustrated in Figure 1, the proteoglycan subunit molecule has structural and functional polarity; one end consists of core protein and predominantly chondroitin sulfate side chains, and the other end, which includes the hyaluronic acid binding site, consists of core proteins and only keratan sulfate side chains (Heinegård and Hascall, 1974). Fragments from either end of the molecule can be obtained by proteolytic digestion of PGS followed by Sepharose or DEAE-cellulose chromatography (Heinegård and Hascall, 1974; Keiser and DeVito, 1974). Immunological analysis of these fragments has revealed that the proteoglycan subunit contains at least two different antigenic determinants, one present in both chondroitin sulfate and keratan sulfate containing peptide fragments and a second found only in keratan sulfate containing peptide fragments (Keiser and DeVito, 1974).

The present report describes the immunodiffusion reactions of antisera to different proteoglycan subunit and PGS fragment preparations with hyaluronidase-digested GPL. It was found that antisera to proteoglycan subunit and fragments derived from it are capable of reacting with the antigenic component of GPL previously associated with the link proteins. These findings suggest that the link proteins may be related to, and perhaps derived from, the hyaluronic acid binding portion of the proteoglycan subunit.

Materials and Methods

Proteoglycan Fractions. PGC, PGS, GPL. Bovine nasal septa were extracted with 4 *M* guanidine and fractionated by successive CsCl density gradients in 0.4 and 4 *M* guanidine, by the method of Hascall and Sajdera (1969), as described in detail previously (Keiser et al., 1972; Keiser and Sandson, 1974). PGC represents the bottom two-fifths of the initial (associative gradient), PGS the bottom two-fifths, and GPL the top fifth of the second (dissociative) gradient.

PPL3. PPL3, derived from a 3 *M* $MgCl_2$ extract of bovine nasal cartilage by the method of Pal et al. (1966), was a gift from Dr. Lawrence Rosenberg.

Keratan Sulfate Fragments. PGS was digested successively with trypsin and chymotrypsin and fractionated by elution from DEAE-cellulose, with this fraction repre-

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[1] Abbreviations used are: PGS, proteoglycan subunit; GPL, glycoprotein link fraction; PGC, proteoglycan complex; PPL3, protein-polysaccharide light fraction, subfraction 3.

senting the eluate from 0.25 to 0.5 *M* KCl, as described in detail previously (Keiser and DeVito, 1974).

Hyaluronic Acid Binding Fragment. As previously described (Heinegård and Hascall, 1974; Keiser, 1975), PGC was digested successively with chondroitinase ABC and trypsin and chromatographed on Sepharose 2B and the initial Sepharose 2B peak was chromatographed on Sephadex G-200 in 4 *M* guanidine. This fraction, which represents the early eluting protein peak from the Sephadex G-200 column, was shown to give a single broad band on dodecyl sulfate gel electrophoresis in our previous paper (Keiser, 1975), where it was designated "2B-1/G200-2".

Preparation of Antisera. Rabbit antisera to PPL3, GPL, PGS, and keratan sulfate fragments were the same reagents used in previous studies (Keiser et al., 1972, Keiser, 1975). Antisera to the hyaluronic acid binding fragment of PGS were prepared as described previously (Keiser et al., 1972). Anti-PPL3, anti-GPL, and anti-PGS were absorbed with lyophilized bovine serum until no precipitin line was noted on immunodiffusion against bovine serum; the antisera to PGS fragments did not require absorption.

Immunodiffusion was performed in 0.6% agarose at pH 8.6 according to standard methods (Keiser et al., 1972). Antisera were concentrated three times by vacuum ultrafiltration. PGS and reduced and alkylated PGS were used as antigens at a concentration of 10 mg/ml, GPL was used at 2 mg/ml, and reduced and alkylated GPL at 4 mg/ml, and each was digested with testicular hyaluronidase before use, as previously described (Keiser et al., 1972). The hyaluronidase used (HSEP grade, Worthington Biochemical Corp., Freehold, N.J.) had no protease activity detectable by immunodiffusion, gel chromatography or dodecyl sulfate gel electrophoresis of hyaluronidase-digested PGS or GPL. PGS digested with chondroitinase ABC (Miles Research Laboratories, Kankakee, Ill.) gave the same immunodiffusion results as hyaluronidase-digested PGS.

Reduction and Alkylation. PGS, 5 mg/ml, or GPL, 4 mg/ml, were dissolved in 0.01 *M* phosphate buffer (pH 6.8). Mercaptoethanol was added to a concentration of 0.01 *M* and the solution was incubated at 37° for 2 hr. Iodoacetate was added to a concentration of 0.02 *M* and incubation at 37°C was continued for an additional 2 hr. The solution was dialyzed against several changes of distilled water and lyophilized.

Results

(1) Link and Subunit Components of GPL. As previously described, immunodiffusion in agarose of antisera to GPL with hyaluronidase-digested GPL yielded two precipitin lines. The line nearer the antibody well fused completely with the single precipitin line formed by anti-GPL and PGS (Figure 2A). Thus GPL appeared to contain two antigenic components, one shared with the proteoglycan subunit and a second due to the link proteins unique to GPL (Keiser et al., 1972).

Antisera to PPL3, a PGS-like fraction prepared by differential precipitation in neutral salt solutions (Pal et al., 1966), gave single precipitin lines upon immunodiffusion with PGS and GPL which fused completely (Figure 2A). This was presumed to represent the reaction of anti-"subunit" antibodies with the "subunit" component of GPL and the absence of antibodies to the link component in these antisera.

(2) Reactions of Antisera to PGS and PGS Fragments. The immunodiffusion reactions with GPL of antisera to PGS and to peptide fragments derived from PGS were inconsistent with the specificity of the link component of GPL suggested in our earlier study.

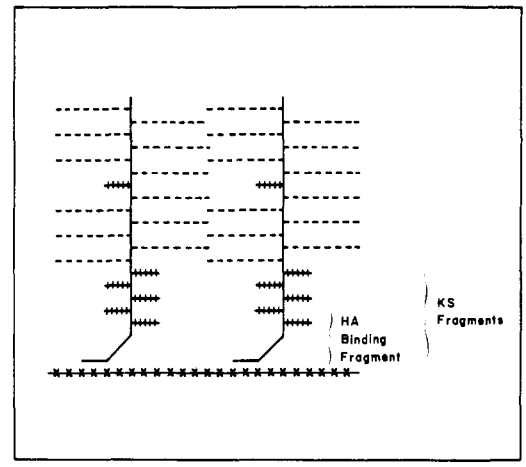

FIGURE 1: Structural model of proteoglycan aggregate. The locations of the trypsin-produced hyaluronic acid (HA) binding fragment (Heinegård and Hascall, 1974) and the keratan sulfate (KS) containing peptide fraction from trypsin + chymotrypsin digests (Keiser and DeVito, 1974) are indicated. The "link proteins" have been omitted. (—) Core protein; (- - -) chondroitin sulfate; (+++) keratan sulfate; (***) hyaluronic acid.

Anti-PGS gave one precipitin line with PGS and, unlike anti-PPL3, two precipitin lines with GPL (Figure 2B). The line nearer the antibody well fused with the precipitin line formed by anti-PGS and PGS, whereas the line nearer the antigen well did not extend beyond the PGS–anti-PGS precipitin line and often appeared to be connected to it by a faint spur (Figure 2B). With the wells appropriately placed, it was found that the line nearer the antibody well formed by GPL and anti-PGS fused with the subunit line formed by GPL and anti-GPL, and the line nearer the antigen well fused with the GPL–anti-GPL link line (Figure 2C). Thus antisera to PGS were capable of reacting with the link component of GPL as well as with the subunit component.

Antisera to keratan sulfate containing fragments of PGS gave a single line upon immunodiffusion with GPL which spurred over the line formed by these antisera and PGS and fused with the link line formed by GPL and anti-GPL (Figure 2D). Under similar conditions, antisera to PPL3 also reacted with the link component of GPL. The absence in Figure 2D of a spur linking the line formed by anti-GPL and PGS with the link line, present in the identical experiments in Figure 2C and E, cannot be explained.

In contrast to the other anti-proteoglycan sera studied, antisera to the hyaluronic acid binding fragment of PGS gave two precipitin lines upon immunodiffusion with PGS (Figure 2E and F). These antisera formed a single line upon immunodiffusion with GPL, which spurred over both lines formed with PGS and fused with the link line formed by GPL and anti-GPL (Figure 2E).

As we have noted in the past (Keiser, 1975), precipitin line spurs are difficult to interpret in this system because of possible quantitative and configurational differences in the antigens of the various proteoglycan fractions.

(3) Reduction and Alkylation. Reduced and alkylated PGS gave only a single precipitin line upon immunodiffusion with antisera to the hyaluronic acid binding fragment of PGS (Figure 2F). All other anti-proteoglycan sera stud-

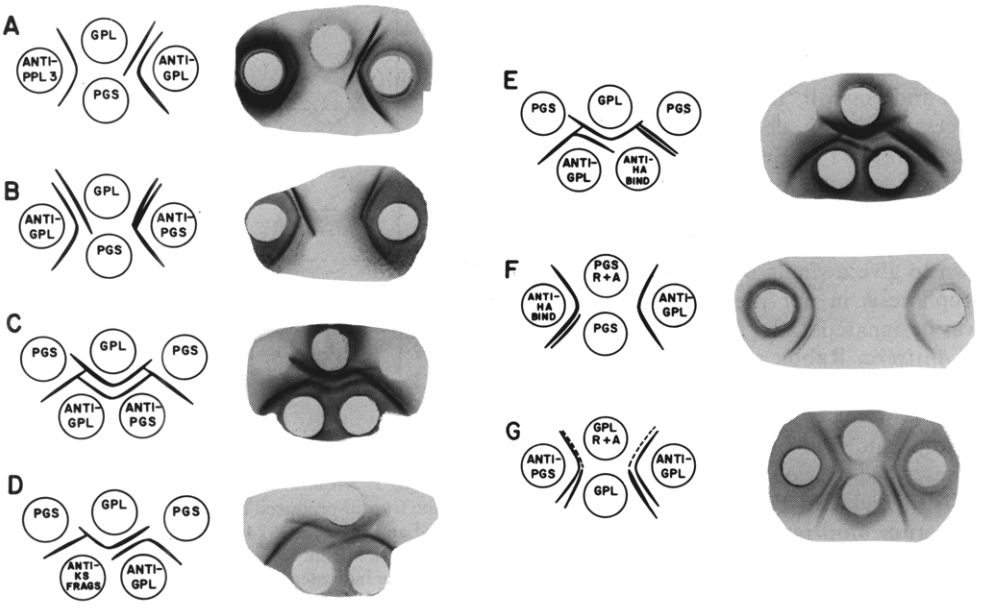

FIGURE 2: Agarose immunodiffusion studies. Wells contained the indicated proteoglycan fractions derived from bovine nasal cartilage or rabbit antisera to the indicated proteoglycan fractions. PGS and reduced and alkylated PGS were used at a concentration of 10 mg/ml, GPL was used at 2 mg/ml, and reduced and alkylated GPL at 4 mg/ml, and each was digested with testicular hyaluronidase. Abbreviations: KS frags, keratan sulfate containing peptide fragments of PGS; HA bind, hyaluronic acid binding fragment of PGS; R + A, reduced and alkylated.

ied appeared to give identical reactions with treated and untreated PGS.

Reduction and alkylation of GPL resulted in a sharp decrease in intensity of the link line formed upon immunodiffusion with anti-GPL compared with GPL similarly incubated and dialyzed in the absence of mercaptoethanol. In addition, after mercaptoethanol treatment of GPL, the link and subunit lines formed with anti-PGS merged into a single or two closely spaced lines (Figure 2G).

Discussion

The results of our earlier investigation of the immunodiffusion reactions of bovine nasal cartilage proteoglycan fractions, using antisera to PPL3, a PGS-like fraction prepared under nondissociating conditions, led us to conclude that proteoglycan aggregates and the GPL fraction contained two antigenic components, one due to the proteoglycan subunit and the second specific for the link proteins (Keiser et al., 1972). However, subsequent experiments reported here, using antisera to dissociatively prepared proteoglycan fractions, indicate that, contrary to our previous conclusion, it is not possible to distinguish between proteoglycan subunit and link proteins on immunological grounds. Thus, in the present study, antisera to PGS were found to react with both link and subunit components of GPL, and antisera to fragments of PGS reacted preferentially with the link component. Reduction and alkylation of GPL resulted in a decrease in the intensity of the link precipitin line formed with anti-GPL sera, indicating that at least one antigenic determinant of the link proteins is dependent on an intact disulfide bond. However, the reaction with anti-PGS sera was also altered by reduction and alkylation of GPL, so that the presence of a sulfhydryl-sensitive antigenic determinant is not unique to GPL. Since antisera to GPL have been shown to react with the two antigenic determinants previously recognized in PGS (Keiser, 1975), it appears that all the antigenic determinants of bovine nasal cartilage proteoglycan thus far identified are present in both GPL and PGS.

In attempting to explain these results, the first possibility to be considered is that the PGS preparations used as immunogens may have been contaminated with GPL. This could arise from an excessively high concentration of proteoglycan in the dissociative CsCl density gradient, as pointed out by Mashburn et al. (1974), coupled with the possibility that the link proteins might be better immunogens in rabbits than the PGS core protein. However, the presence of link proteins in the PGS preparations used for immunization could not be detected by immunodiffusion at concentrations as high as 20 mg/ml or by dodecyl sulfate polyacrylamide gel electrophoresis of as much as 3 mg. In addition, fractions of individual link proteins isolated from GPL (kindly provided by Dr. John Gregory) have proven unexpectedly poor immunogens (unpublished observations). Furthermore, as shown in Figure 2B, the anti-"link" antibodies in anti-PGS sera and those in anti-GPL sera react differently on immunodiffusion with GPL. Thus, GPL contamination of PGS would seem to be a highly unlikely explanation for these immunodiffusion results.

A far more reasonable explanation for the immunological results presented here is that the proteoglycan subunit and the link proteins share antigenic determinants. Although the proteoglycan subunit and link proteins may be distinct structural and functional elements of proteoglycan aggregate, they may nonetheless have primary structural features and antigenic determinants in common, perhaps on the basis of a common evolutionary origin.

Another possibility consistent with the findings described here is that the link proteins are identical with and derived from the hyaluronic acid binding portion of the proteoglycan subunit molecule. If so, PGS preparations should contain "defective" proteoglycan subunit molecules, lacking the link protein portion, in addition to intact proteoglycan subunits. The presence of both types of subunits could explain why two precipitin lines are formed upon immunodiffusion of PGS with antisera to the trypsin-produced hyaluronic acid binding fragment of the proteoglycan subunit (Figure 2E and F). Also, the difference in reactivity with GPL of antisera to PPL3 and PGS (Figure 2A and B) may

be due to PPL3 having been isolated under nondissociative conditions resulting in the presence of a greater proportion of "defective" subunits than in PGS. In addition to explaining the immunological findings, this hypothesis provides a basis for understanding why both the proteoglycan subunit and the link proteins bind specifically to hyaluronic acid (Heinegård and Hascall, 1974) and why both require intact disulfide bonds for aggregate formation (Hascall and Sajdera, 1969). Finally, the proteoglycan molecules of smaller size and different chemical composition from proteoglycan subunit found in cartilage extracts (Mayes et al., 1973; Hardingham and Muir, 1974) might result from the same degradative process which produces the link proteins.

Two types of observations seemingly argue against the hypothesis that the link proteins are identical with a portion of the proteoglycan subunit. First, Heinegård and Hascall (1974) showed that a protein with the mobility of one of the link proteins on dodecyl sulfate polyacrylamide gel electrophoresis was derived from ^{3}H-acetylated GPL and not from ^{3}H-acetylated PGS. Their experiment involved treatment of labeled reaggregated proteoglycan fractions with trypsin and, in fact, proved only that under the experimental conditions employed link proteins could not be derived from PGS by trypsin digestion. Second, it is clear that proteoglycan aggregates formed by the combination of PGS and GPL are more stable than aggregates formed by PGS and hyaluronic acid in the absence of link proteins (Gregory, 1973; Hascall and Heinegård, 1974a,b). However, the fact that the link proteins may have a functional role need not imply anything about their structure or origin.

Since the precise nature of the antigenic determinants of cartilage proteoglycan have not been elucidated and monospecific antisera are not available, it is not possible on the basis of immunological data alone to determine whether the link proteins are identical with a portion of the proteoglycan subunit or are distinct structures related in part to the proteoglycan subunit. The relationship between the link proteins and the proteoglycan subunit could be more conclusively defined by comparing their amino acid sequences or by deriving link proteins from PGS in vitro. Studies currently in progress suggest that enzymatic digestion of PGS can yield fragments similar, though not identical, in dodecyl sulfate polyacrylamide gel electrophoretic mobility and immunodiffusion properties to naturally occurring link proteins.

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