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Interaction of Cartilage Proteoglycans With Hyaluronic Acid

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Most proteoglycans are present in hyaline cartilage matrices as aggregates with as many as 100 molecules, each with average molecular weight of about 2×10^6, bound through specific, noncovalent interactions to individual strands of hyaluronic acid (HA). The interactions with HA are mediated by the HA-binding region of the core protein, which is located at one end of each of the interactive proteoglycans. A fragment of the core protein, average molecular weight of about 6×10^4, which contains the HA-binding site, can be isolated in an active form from trypsin digests of proteoglycan aggregates. The "active" HA-binding site in this preparation interacts strongly with HA-10 but weakly with HA-8, (oligomers of HA derived from partial digests of HA with testicular hyaluronidase); HA-9 derived from β-glucuronidase digestion of HA-10 also interacts strongly. No polysaccharide other than HA has been found to interact. Christner, Brown, and Dziewiatkowski (personal communication) modified the carboxyls on glucuronic acid groups in a mixture of HA-10 to HA-30, and they found that the interaction with proteoglycan no longer occurred if about 60% of the total carboxyls were a) methyl esterified, b) reduced to glucose, or c) substituted with glycine in amide linkage. Saponification of the methyl esters restored activity. Dansylation of lysine residues in the HA-binding region preparation abolished binding activity. However, when the dansylation reaction was done in the presence of HA, the HA-binding activity was protected. Acetylation of the same residues did not abolish binding activity but did prevent subsequent inactivation by dansylation. Hardingham, Ewins, and Muir (Biochem J 157:127–143, 1976) studied the effect of various amino acid modifiers on the interaction of intact proteoglycans with HA and showed that reaction of arginine residues with low concentrations of 2,3-butanedione was particularly effective in destroying binding. In sum, the data above suggests that the HA-binding region a) contains accessible arginine residues necessary for activity, b) contains lysine residues near the binding site which, when substituted with bulky groups such as dansyl, but not acetyl, sterically block interaction, and c) requires a length of HA with at least 4.5 repeat disaccharides containing 3, and possibly 4, unmodified glucuronic acid carboxyls for interaction. The possible relevance of proteoglycan-hyaluronic acid interaction to the observations that hyaluronic acid specifically inhibits proteoglycan synthesis by cultured chondrocytes is discussed.

Key words: proteoglycans, cartilage, hyaluronic acid

I. STRUCTURE AND FUNCTION OF CARTILAGE PROTEOGLYCANS

Proteoglycans are structural components of the extracellular matrix of hyaline cartilages. These macromolecules consist of a core protein structure to which a large number of chondroitin sulfate (CS) and keratan sulfate (KS) chains are covalently attached.

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The average proteoglycan molecule from bovine nasal cartilage, for example, has a molecular weight of approximately 2.5×10^6 (1, 2). It contains a core protein of about 200,000 molecular weight with about 100 chondroitin sulfate chains, each with an average molecular weight of 20,000, and 30–60 keratan sulfate chains, each with molecular weight of 4,000–8,000, distributed along specialized regions of the core protein (Fig. 1, Refs. 1–5). A large proportion of the proteoglycans have a portion of protein, the hyaluronic acid (HA)-binding region, located at one end of the core (6–8). (See section II below.) About 65% of the keratan sulfate chains are localized on another portion of the core, the KS-enriched region, adjacent to the HA-binding region, while more than 90% of the chondroitin sulfate chains are attached to the CS-enriched region, located further from the HA-binding region (Fig. 1, Refs. 5, 9). The family of proteoglycan molecules in bovine nasal cartilage is, however, widely polydisperse with molecular weights ranging from a few hundred thousand to more than 4 million (1). This range of molecular weights is primarily the result of a variation in the number of chondroitin sulfate chains bound to each core protein (1, 9–11); it is, moreover, correlated with changes in the overall lengths of proteoglycan cores observed in electron microscopic studies of proteoglycans spread on thin films of cytochrome c (12, 13), and also with amino acid and hexosamine changes which suggest that polypeptide in the CS-enriched region is longer when it contains more chondroitin sulfate chains (9–11).

In the molecular architecture of a proteoglycan molecule, the polysaccharide chains are constrained by being attached at one end to the core protein. For this reason, the intact macromolecules have hydrodynamic properties which are different from those of

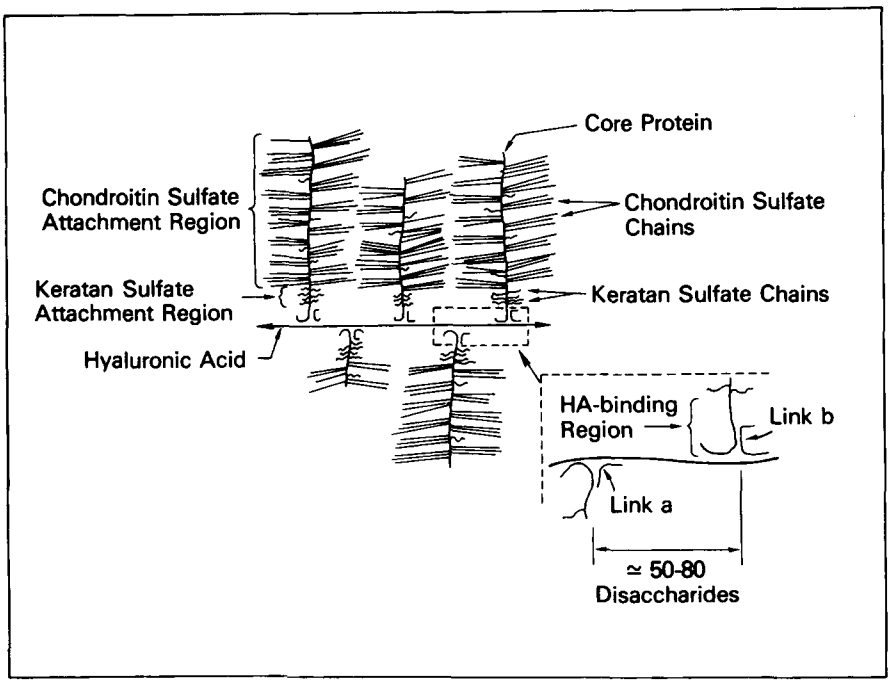


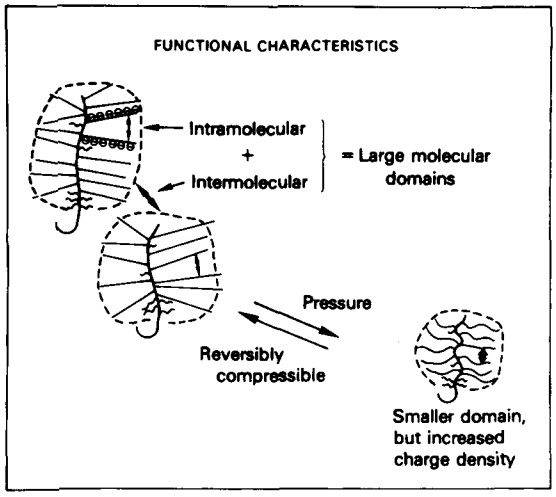
Fig. 1. Schematic model for the structure of cartilage proteoglycans and the interactions involved in the formation of aggregates.

the isolated polysaccharide chains. The highly anionic chains extend out from the core and, hence, the macromolecules occupy large molecular domains which encompass large amounts of solvent per mass of proteoglycan. The high limiting viscosity values for proteoglycans indicate that the molecules occupy solution volumes 30–50 times their dry weight (1, 2) and, for proteoglycans isolated from bovine nasal septa, have radii of gyration of almost 600 Å in 0.5 M guanidinium chloride (2).This property of intact proteoglycans is critical for the structure of cartilages. For example, the proteoglycans occupy large volumes in the extracellular matrix and provide a network for the retention of large amounts of solvent. Further, the proteoglycans can be compressed reversibly by displacing solvent from the molecular domain at the expense of increasing intramolecular interactions.

This is shown by the results of the experiment depicted in Fig. 2. Monomer (PGS) and aggregate (PGC) solutions were pelleted in the ultracentrifuge and the equilibrium packed volumes measured at different centrifugal speeds (14). For the lowest speeds, values measured at the beginning and end of the experiment coincided, indicating that the molecules were reversibly compressible. The data also indicate that under these experimental conditions, the limiting packed solute volume (extrapolating $1/\omega^2$ to zero) is between 25–30 ml/g. The concentration of proteoglycans in most hyaline cartilages is such that the largest volume they could possibly occupy in vivo would be far less than these values, and they must therefore occupy smaller domains in the tissue than their extended conformations occupy in solutions.

These physical chemical properties of the proteoglycans provide cartilages with their essential properties of resiliency and stiffness. The potential consequences for cartilages of impaired proteoglycan function have been demonstrated in several systems. Chemical (15, 16) and morphological (16) studies of cartilages in the mutant nanomelic chicken have revealed a virtual absence of cartilage specific proteoglycans, but normal amounts of type II collagen, in cartilage matrices. As a consequence, the chondrocytes are much closer together in the matrix than is normal (Fig. 3). The deficiency causes severe skeletal deformities and is lethal, usually before hatching. The classic experiments of Thomas (17) showed that intravenous injections of crude papain into young rabbits caused depolymerization of proteoglycans in cartilage matrices. This lead to a loss of tissue function, most notably in the collapse of the ear cartilages. The proteoglycans in the epiphyseal cartilages of brachiomorphic mice have been shown to be significantly undersulfated in comparison with epiphyseal cartilage from normal mice (18). Because of the reduction in charge density, there would be a decrease in the intramolecular interactions in the proteoglycan molecules with a concomitant reduction in the sizes of their molecular domains. This may account for the smaller size observed for the epiphyses in these animals and the striking foreshortening of their limbs. Increased proteolysis of proteoglycans in many osteoarthritic lesions leads to increased cartilage penetrability and loss of cartilage resiliency, (see Ref. 19 for a recent review).

Although some of the proteoglycans are apparently present in the extracellular matrices of cartilages as individual molecules, most (60–85%) are present as very large aggregates in most cartilaginous tissues (7, 12, 20–24). The function of these aggregate structures for the tissue is not known, but they may be important for immobilizing the proteoglycans and providing a more ordered structure within the matrix. The central filament of each aggregate is provided by a strand of hyaluronic acid of variable length (Fig. 1) as was first suggested by the work of Hardingham and Muir (6) (see section III below). Interactive proteoglycan monomers bind at intervals along the hyaluronic acid by means of a highly specific interaction mediated by the HA-binding region of the core protein (8).



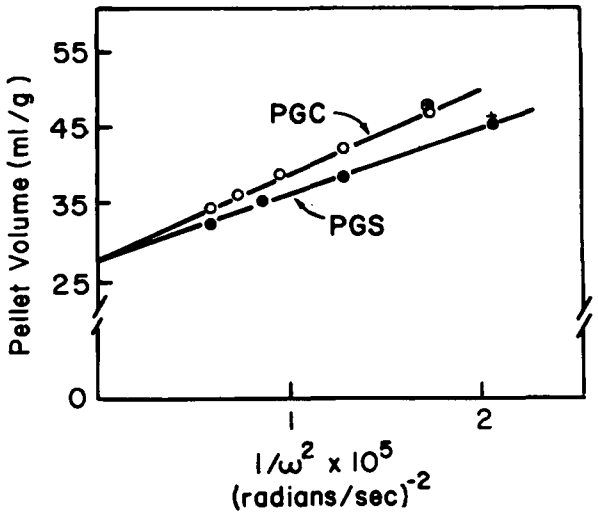


Fig. 2. a) Schematic model illustrating the functional characteristics of the intact cartilage proteoglycans. b) Compressibility of proteoglycans in centrifugal fields. Monomer (PGS) and aggregate (PGC) solutions, 2.4 mg/ml and 3.0 mg/ml respectively, in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8, were centrifuged in an analytical ultracentrifuge at different speeds. The equilibrium packed volumes of the solute were measured for each speed and are plotted against the reciprocal of the angular momentum squared, $(1/\omega^2)$. The plus symbols indicate values for the packed volumes at the lowest speeds which were observed at the end of the experiment after the other speeds were studied. The fact that they coincide with the first measurements made at those speeds indicates that the compressibilities of the solutes were reversible. The packed volumes observed for a preparation of papain digested monomer in a similar experiment were much smaller and difficult to measure reliably indicating that the hydrodynamic volumes of the intact proteoglycans are larger than those of equivalent amounts of the free glycosaminoglycan chains. Data taken from Ref. 14.

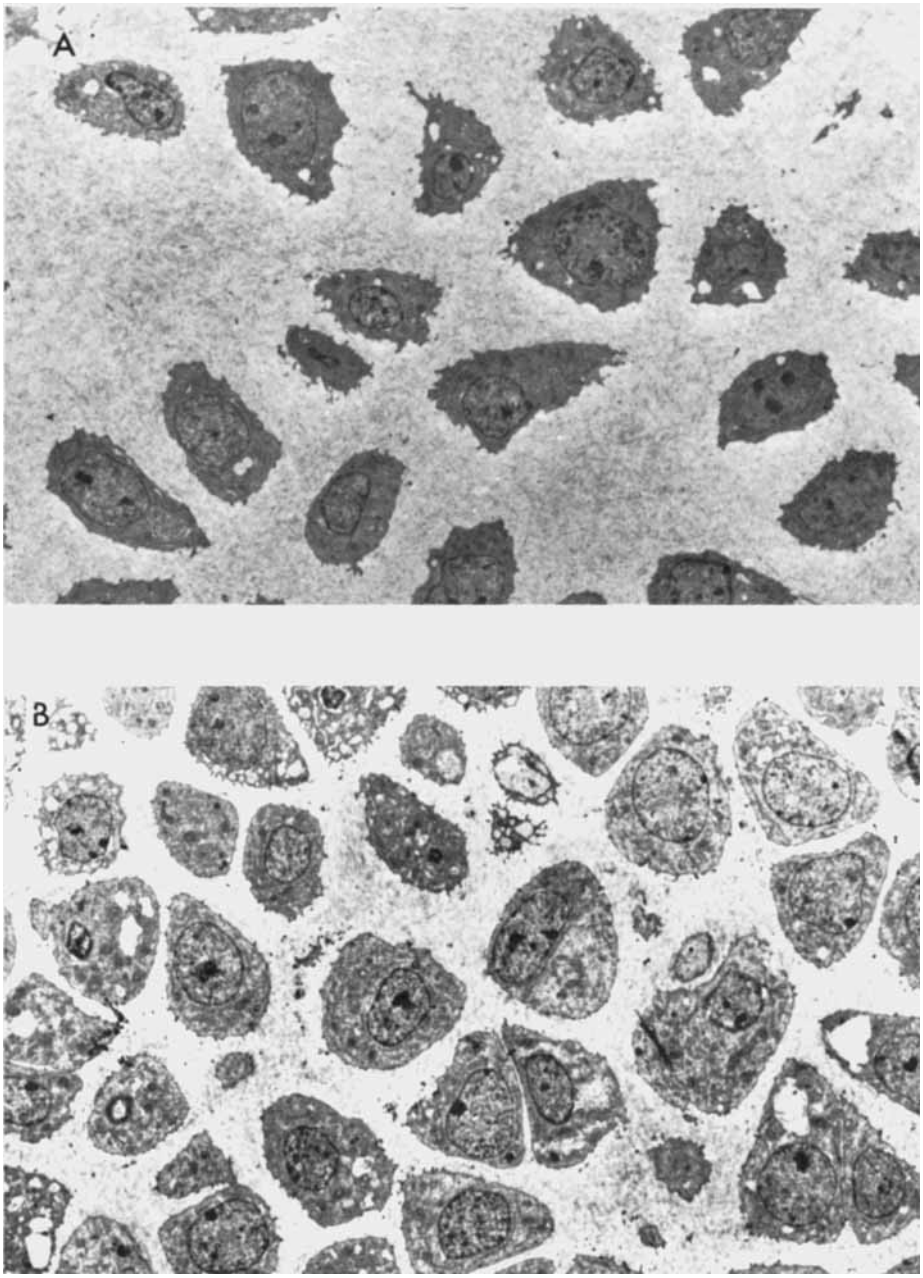


Fig. 3. Low power electron microscope pictures of sections of sternal cartilage from A) normal chick embryos and B) nanomelic chick embryos. Printed with the kind permission of Dr. Jack Pennypacker; see Ref. 16.

The average sizes of the aggregates appear to depend upon the length of the hyaluronic acid filament and can vary for different cartilages (7). Aggregates with over 100 monomers have been observed in the electron microscope (12). Two small proteins (molecular weights of 42,000 and 50,000), referred to as link a and link b respectively, are present in the structure (7, 22, 25, 26). They are capable of interacting with hyaluronic acid in the absence of proteoglycans (8), and they probably interact directly in some manner with the HA-binding region protein (Fig. 1).

II. ISOLATION OF HA-BINDING REGION PROTEIN

While interactive proteoglycan molecules will bind to hyaluronic acid in the absence of the link proteins (6), the presence of these low-molecular-weight components in the structure of the aggregate provides additional stability to the interactions (26). This enhanced stability was critical for the successful isolation of a functional HA-binding region protein preparation from intact proteoglycan aggregates. Heinegard and Hascall (8) observed that a high-molecular-weight complex consisting of hyaluronic acid and 2 associated proteins could be recovered from aggregate preparations which were digested first with chondroitinase and then trypsin. When the same experiment was done with reconstituted aggregates which contained either ^{3}H-acetylated monomer proteoglycans or ^{3}H-acetylated link protein components, it was shown that the larger of the 2 proteins associated with the hyaluronic acid was derived from the protein core of proteoglycan molecules while the smaller was derived from the link proteins. However, when a mixture of purified monomer proteoglycans with hyaluronic acid was treated with trypsin, no protein component remained bound to the hyaluronic acid. The HA-binding region preparation recovered from the trypsin digest of aggregate contained some bound keratan sulfate which accounted for the wide, continuous range of apparent molecular weights, average of about 90,000, that was observed for this preparation on sodium dodecyl sulfate-polyacrylamide gels in the presence or absence of sulfhydryl reducing agents (8). The actual size of the protein moiety in the HA-binding region appears to be smaller. Mild papain digestion of aggregates yielded a fragment from the HA-binding region of about 65,000 which contained little or no keratan sulfate (7) and trypsin digestion of aggregates isolated from the Swarm rat chondrosarcoma, which contain no keratan sulfate, yielded a HA-binding region preparation with molecular weight of about 67,000 (23, 27). More recently it has been shown that the 2 link proteins in aggregates are related to each other, with the larger link b molecules containing a glycopeptide extension which is absent from the link a molecules. This was shown by the results of the experiment depicted in Fig. 4. Sodium dodecyl sulfate-polyacrylamide electrophoretic gels were prepared from identical aliquots of aggregate samples which had been treated either with chondroitinase ABC alone or with the chondroitinase and then trypsin. After chondroitinase treatment alone, both the link b and link a molecules were present. After the subsequent trypsin treatment, the link b molecules electrophoresed with the link a molecules. This was indicated by the absence of the link b band and the proportional increase in staining intensity of the link a band. Further, Baker and Caterson (28) have provided evidence that a proportion of the link b molecules are converted to link a after treatment with sulfhydryl reducing reagents and that the extra portion of polypeptide on link b contains carbohydrate. It may be that link a is the end product of a sequence of proteolytic modifications that take place during the normal metabolism of proteoglycan aggregates. It is of interest that only link a is present in proteoglycan aggregates

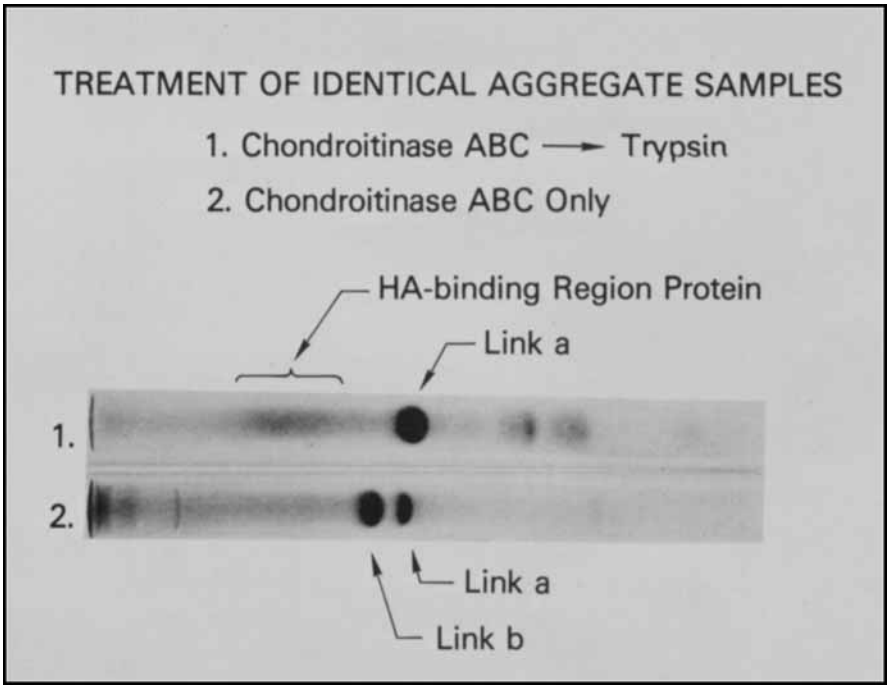


Fig. 4. Samples were treated with the enzymes indicated above as described elsewhere (8). After dialyses and lyophilization, identical quantities (about 25μg) were electrophoresed on 7% polyacrylamide gels and stained with Coomassie Blue (7).

isolated from a transplantable rat chondrosarcoma which contains high levels of proteolytic activity (23).

The above results suggest that interactions between a) the HA-binding region protein and hyaluronic acid, b) the link protein and hyaluronic acid, and c) the HA-binding region protein and the link protein, are all involved in the organization of the aggregate structure, Fig. 1.

A procedure adapted from the results of Heinegard and Hascall (8) can be used to purify the HA-binding region protein and the link protein (Ref. 5, Fig. 5). Trypsin digests of aggregate preparations are fractionated in an associative density gradient. The chondroitin sulfate peptides have high buoyant densities and are recovered in the bottom of the gradient, whereas the complex of hyaluronic acid and associated proteins has a much lower buoyant density and is recovered in the top of the gradient. The top fraction is chromatographed on Sepharose 2B to purify the complex from enzyme and peptide fragments. The individual components of the complex can subsequently be separated by chromatography on Sephadex G 200 in the presence of a dissociative solvent, 4 M guanidinium chloride. The high-molecular-weight hyaluronic acid elutes near or partially in the void volume while the HA-binding protein and the link protein are resolved separately as partially included peaks (Fig. 5). The purified HA-binding-region protein fraction, which still contains some bound keratan sulfate, is soluble in associative solvents and was shown to interact efficiently with hyaluronic acid (8) (see section IV below). The purified link

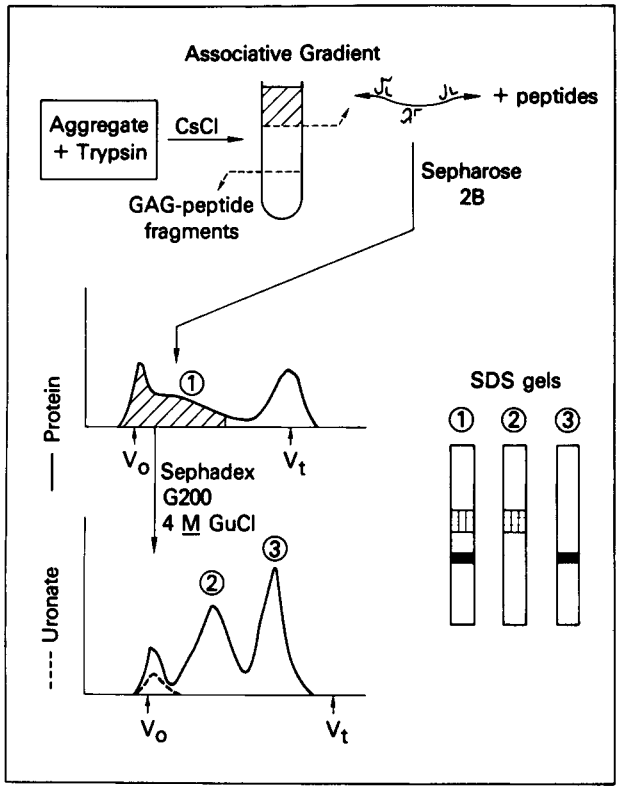


Fig. 5. Schematic outline of the procedure presently used to isolate the HA-binding-region protein and the link a protein. The elution profile on Sepharose 2B is used with kind permission of Dr. Dick Heinegard. See Ref. 8.

protein was shown to be capable of interacting with hyaluronic acid (8); however, by itself, the preparation is insoluble in most associative solvents. In recent experiments, however, it has been shown that the link protein, purified by the procedure outlined in Fig. 5, can be kept soluble in a solvent including 1 M LiCl and 0.15 M guanidinium chloride (R. A. Gelman and V. C. Hascall, unpublished observations). In this solvent the molecules still interact effectively with hyaluronic acid. Thus, it should now be possible to determine the specificity of the interaction between link protein and hyaluronic acid in a similar manner as for the HA-binding-region protein discussed below.

III. MODIFICATION OF HYALURONIC ACID

In a series of experiments that were crucial for determining the mechanism of proteoglycan aggregation, Hardingham and Muir (6) showed that in associative solvents, a large proportion of the monomer proteoglycans isolated from pig laryngeal cartilage interacted specifically with small amounts of hyaluronic acid, a glycosaminolgycan with a repeating disaccharide structure of $[(1\rightarrow4)$-β-glucuronosyl-$(1\rightarrow3)$-β- N-acetylglucosaminosyl$]_n$ (see Figs. 9 and 10 below). The viscosities of mixtures of the proteoglycan fraction with a preparation of umbilical cord hyaluronic acid of average molecular weight 500,000 increased up to a maximum in mixtures with slightly less than 1% (wt/wt) hyaluronic

acid. These results indicated that the effective hydrodynamic sizes of the proteoglycans increased in the presence of hyaluronic acid, suggesting that aggregation was occurring. This was verified by chromatography of mixtures on Sepharose 2B; more than 50% of the proteoglycan molecules shifted from the broad, included peak observed in the absence of hyaluronic acid, into the column void volume in mixtures with 0.5–1.0% hyaluronic acid. Calculations using approximate molecular weights suggested that at maximum saturation of available hyaluronic acid, bound proteoglycans were an average of about 120 HA-monosaccharide units (60 repeat disaccharides) apart. Other polyelectrolytes, dextran sulfate, chondroitin sulfate, sodium alginate, and DNA, did not interact with proteoglycans. Further, the proteoglycan interaction with hyaluronic acid was reversed when the solution pH was lowered to pH 3–4 and when the solution concentration of guanidinium chloride was raised to 1–2 M. Since these conditions had been shown to dissociate intact proteoglycan aggregates (21, 22), Hardingham and Muir (6, 20) suggested that hyaluronic acid might be an important component of the aggregation mechanism.

Hascall and Heinegard (29) subsequently purified a series of HA-oligomers, with multiples of the repeat disaccharide, from partial digests of hyaluronic acid with testicular hyaluronidase. These oligomers were tested for their ability to interact with intact proteoglycans isolated from bovine nasal cartilage (29) and pig laryngeal cartilage (30). A competitive viscosity assay such as that indicated in Fig. 6 was used (30). When 1% (wt/wt) of a hyaluronic acid preparation was added to a monomer proteoglycan solution, the viscosity of the mixture increased to a higher, plateau value within 45 min (Fig. 7a). At that time, aliquots with various concentrations of HA-oligomers were added to equivalent mixtures of proteoglycan with hyaluronic acid, and changes in the solution viscosities

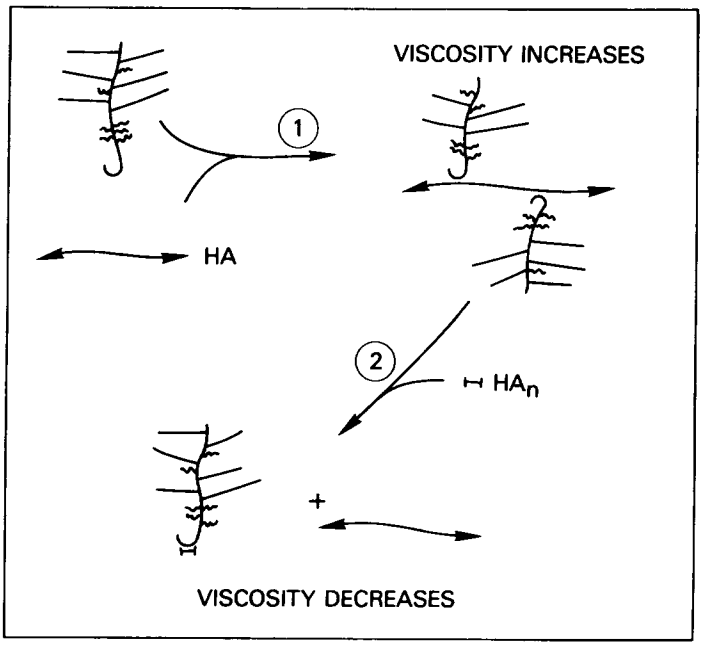


Fig. 6. Schematic outline of the procedure used to test HA-oligomers of various sizes for their ability to compete with intact hyaluronic acid for binding with proteoglycans.

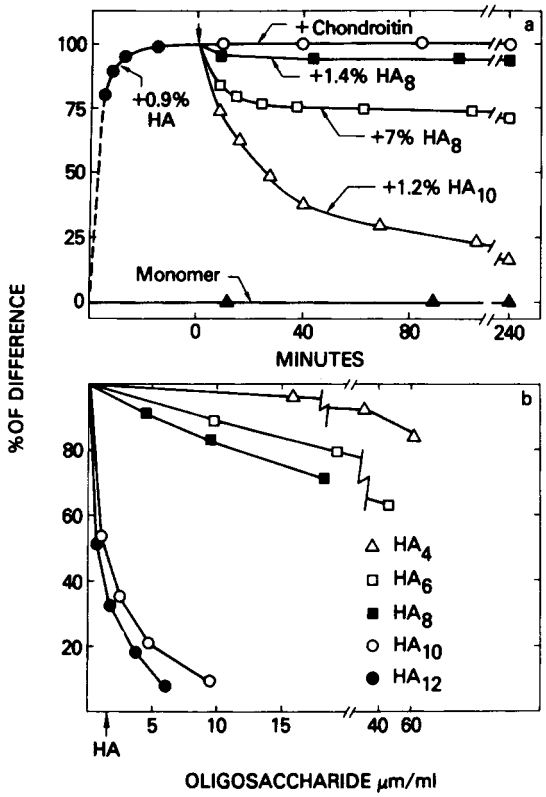


Fig. 7. The results of experiments a) from Ref. 29 and b) from Ref. 30 (with the kind permission of Drs. Timothy Hardingham and Helen Muir) which indicate the strong interactivity for binding of HA-oligomers greater than or equal to HA_{10}. See text for details.

were observed. When the HA-oligomers were capable of displacing the bound proteoglycans from intact hyaluronic acid molecules by competing for the binding site, the solution viscosities decreased. After several hours, equilibrium viscosity values intermediate between the proteoglycan solutions with and without 0.9% hyaluronic acid were observed. These values provided a measure of the equilibrium amounts of proteoglycan bound either to the HA-oligomers or to the intact molecules of hyaluronic acid. The experiments showed a striking difference between the effectiveness of binding of the HA_8 and HA_{10} oligomers (4 and 5 repeat disaccharides, respectively). A 1.2% concentration of HA_{10} decreased the viscosity to a much lower equilibrium value, about 17% of the difference, than did a much higher, 7%, concentration of HA_8 (Fig. 7a). When different concentrations of the oligomers were tested by Hardingham and Muir (30), the equilibrium viscosity levels of equimolar mixtures of HA-oligomers with intact hyaluronic acid were only 40% of the difference for HA_{10} but greater than 95% for HA_8 (Fig. 7b). Chondroitin, a structural analogue of hyaluronic acid in which the N-acetylglucosamine residues are replaced by N-acetylgalactosamine, did not displace proteoglycans from hyaluronic acid at all, and therefore did not interact with the binding site (Fig. 7a). A related polysaccharide, an unsulfated N-acetylated intermediate in the biosynthesis of heparin (31), also is not able to interact with proteoglycans (Heinegard, personal communication). This polysaccha-

ride contains the same sugar moieties, N-acetyglucosamine and glucuronic acid, as hyaluronic acid but the glycosidic linkages are different [(1→4)-α-glucuronosyl-(1→4)-β-N-acetyl-glucosaminosyl]$_n$ from those in hyaluronic acid. While oligomers of HA_{10} or greater are effective in displacing proteoglycan monomers from hyaluronic acid in mixtures, they are ineffective in displacing proteoglycans from intact aggregates under similar experimental conditions (29). This provides further evidence that the link proteins add stability to the aggregates.

These experiments suggested that the highly specific interaction between the proteoglycan molecules and hyaluronic acid was mediated by a portion of the core protein in the interactive proteoglycan molecules. Evidence for this was provided when it was shown that proteoglycan core molecules, in which the chondroitin sulfate chains were enzymatically removed by chondroitinase digestion, still interacted with hyaluronic acid with the same specificity (29). Mixtures of this core preparation with different amounts of hyaluronic acid were chromatographed on Sepharose 2B in an associative solvent. The elution profiles indicated that more than 70% of the core molecules were able to bind to hyaluronic acid (Fig. 8). Further, at saturating levels of core to hyaluronic acid, each bound core molecule sterically occupied about 24 HA-monosaccharide units, (12 repeat disaccharides). This was in contrast to intact proteoglycans where the available data, including the stoichiometry of mixtures of proteoglycan with hyaluronic acid (6), the proportion of hyaluronic acid in aggregate preparations (7, 20), and electron microscopy (12), indicate that intact proteoglycan molecules are located a minimum of 80–120 HA-monosaccharide units apart. In many cases, therefore, the packing density of intact proteoglycans along hyaluronic acid in aggregates, appears to be limited by the exclusion volume of the monomers, which is primarily a function of the number and length of the attached chondroitin sulfate chains. Additional experiments in which different HA-oligomers were added to mixtures of core molecules in the presence of intact hyaluronic acid revealed that HA_{10} but not HA_8 was effective in displacing core molecules (Fig. 8), and that HA_9, an oligomer derived from β-glucuronidase digestion of HA_{10}, was almost as effective as HA_{10} (29).

Subsequent experiments indicated that an intact polypeptide fraction, the HA-binding-region protein, could be recovered from the core structure of interactive proteoglycan molecules as discussed in section II above. All of the molecules in the HA-binding region preparation were able to interact with hyaluronic acid (8). As with the core molecules, the minimum packing distance of the HA-binding-region proteins at saturation of available hyaluronic acid was about 20–24 HA-monosaccharides apart. This suggests that the keratan sulfate and the bulk of the polysaccharide attachment region protein which are present in the core, but not in the HA-binding region molecules, do not sterically interfere with the molecular packing along the hyaluronic acid molecule.

Recently, Christner, Brown, and Dziewiatkowski (32) used several chemical procedures to modify variable proportions of the carboxyl groups on glucuronic acid residues in a mixture of HA-oligomers (from HA_{10} to HA_{30}) derived from partial digests of hyaluronic acid with testicular hyaluronidase (see Fig. 9). Subsequently, the modified HA-oligomers were tested for competition with intact hyaluronic acid in an equilibrium viscosity assay similar to that described above (Fig. 6). Initially, essentially all of the carboxyl groups in a sample of the HA-oligomers were methylesterified by reacting them with diazomethane. Samples were then partially saponified to generate a series of oligomers in which the proportion of methylglucuronate to unsubstituted glucuronate groups varied. Other samples of the completely esterified preparation were treated to different extents

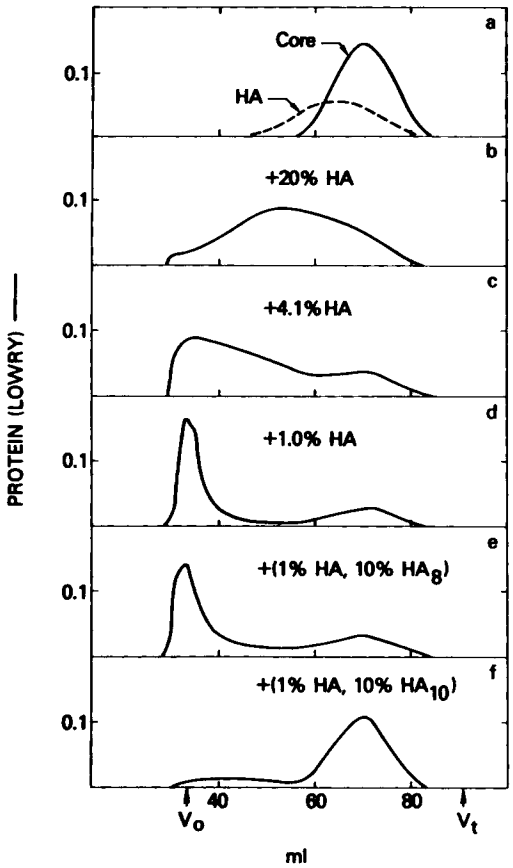


Fig. 8. A series of experiments in which the interaction of proteoglycan core molecules (isolated from chondroitinase ABC digests of monomer proteoglycans) with hyaluronic acid and HA-oligomers was tested. The dashed curve a) indicates the elution profile determined in a separate experiment of the intact hyaluronic acid sample used in all subsequent experiments (b–f). Taken from Ref. 29. See text for details.

with sodium borohydride to reduce a proportion of the methylglucuronate residues to glucose. After these samples were then completely saponified, a second series of oligomers was generated in which the proportion of glucose to glucuronate groups varied. A third set of modifications on a separate sample of the HA-oligomers involved the addition of variable amounts of the methyl ester of glycine by carbodiimide facilitated amide linkage to glucuronic acid carboxyl residues. The methyl esters on the glycines were subsequently saponified in some cases (Fig. 9). All of these series of substitutions gave similar results in the competition assay. When the proportion of free carboxylate groups to substituted or modified moieties was less than about 40% of the total, the modified oligomers showed little or no competition with intact molecules of hyaluronic acid for binding to proteoglycan and, therefore, were unable to interact with the HA-binding site. As the proportion of the unmodified glucuronate carboxyl groups increased toward 100%, competition increased approximately linearly to greater than 90% of that exhibited by the original, unsubstituted HA-oligomer sample. A statistical analysis of the results suggested that effective interaction with the HA-binding site required (probably at least 3 of

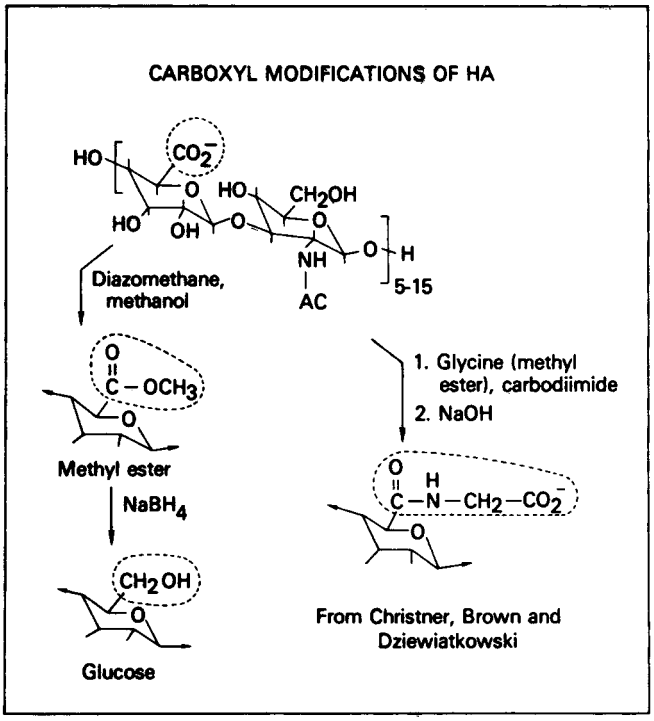


Fig. 9. Schematic outline of the reactions used to modify the carboxyl groups of HA-oligomers, Ref. 32.

the 4) unmodified carboxyl groups on the glucuronic acid residues in that portion of hyaluronic acid which is bound in the active site.

The experiments above indicate that the HA_8 oligomer derived from partial testicular hyaluronidase digestion of hyaluronic acid does not interact strongly with the HA-binding site of interactive proteoglycan molecules. The presence of an additional N-acetylglucosamine residue at the nonreducing end to give the HA_9 oligomer increases the strength of the interaction greatly (Fig. 10). It remains to be determined if the iso-HA_8-oligomer, in which glucuronic acid is at the reducing end and which could be prepared from partial digests of hyaluronic acid with leech hyaluronidase, is sufficient for strong binding. This iso-HA_8-oligomer, then, would be the minimum possible length of hyaluronic acid required for strong interaction, indicating that the active binding site in the protein core extends over at least 4 repeat disaccharides with N-acetylglucosamine at the nonreducing end (Fig. 10). At least 1, and probably more, of the N-acetylglucosamine residues are required in the active site interaction since chondroitin, in which the N-acetylglucosamines are replaced by N-acetylgalactosamines, does not interact. Finally, it is likely that as many as 3 of the 4 carboxyl residues of the glucuronic acid moieties in the active site must have unsubstituted carboxyl groups. It is also possible that protonation of the carboxyl groups is the explanation for the reversal of the interaction between proteoglycans and hyaluronic acid observed at solution pH values between 3 and 4 (6, 20, 22), because this is the region for the dissociation constants of these groups in hyaluronic acid (33).

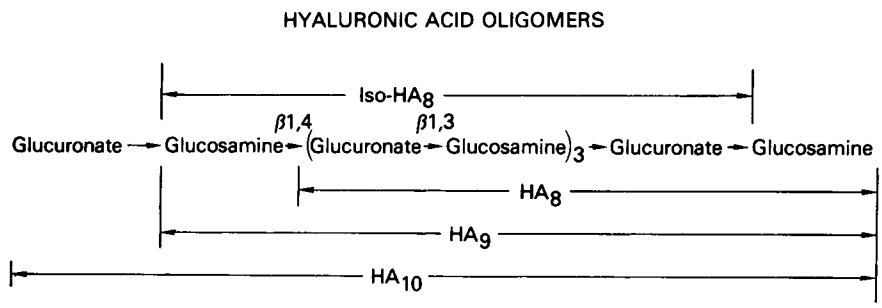


Fig. 10. Structure of various HA-oligomers.

The work of Highsmith et al. (34) on the interaction of hyaluronic acid with bovine testicular hyaluronidase has shown that the active site of this enzyme has 5 subsites for hyalobiuronate residues, the repeat disaccharide of hyaluronic acid. Thus, the enzyme interacts strongly with HA_{10} and less strongly with shorter oligosaccharides, producing primarily HA_8-, HA_6-, and HA_4- oligomers under conditions of complete digestion. The interaction of hyaluronic acid with hyaluronidase is unlike that of the interaction with the HA-binding region of the proteoglycan in 2 major respects; first the hyaluronidase hydrolyzes the hyaluronic acid, and second, it interacts with and digests chondroitin-4- and -6-sulfates as well.

IV. MODIFICATION OF THE HA-BINDING REGION PROTEIN

Sajdera and Hascall (21) showed that reduction and alkylation of disulfide groups in monomer proteoglycans prevented subsequent aggregate formation. This suggested that the conformation of the protein portion of the proteoglycan molecule was critical for the aggregation mechanism. Subsequently, it was shown that reduced and alkylated proteoglycan monomers would not interact with hyaluronic acid (6) and that reoxidation of reduced, but not alkylated, monomers restored almost 90% of the interactivity (10). Hardingham, Ewin, and Muir (10) used a variety of reagents to modify specific amino acid residues within the protein portion of proteoglycan monomers. The modified samples were then tested for their ability to interact with hyaluronic acid. The results from this study are summarized in Table I. The interaction with hyaluronic acid was inhibited after the proteoglycan preparation was treated with sufficient concentrations of reagents which modify primarily a) lysine groups (acetic anhydride, 2-methylmaleic anhydride), b) arginine groups (2,3-butanedione), or c) tryptophan groups (2-nitrophenylsulfonyl chloride). Treatment of proteoglycans with 0.055 M and 0.55 M 2-methylmaleic anhydride caused the loss of 67% and 100% of their binding capacity respectively. In the latter case most of the 2-methylmaleyl groups could be removed by treating the substituted proteoglycans with dilute acid with recovery of 60% of the binding activity. Up to two-thirds of the arginine residues in the proteoglycan could be modified by reaction with 2,3-butanedione without altering the interaction with hyaluronic acid; but with further arginine modification the interaction was almost completely abolished (Table I). Modification of about one third of the tryptophan with 2-nitrophenylsulfonyl chloride also eliminated most of the binding activity. Fluorescence measurements on the proteoglycan in an associative solvent suggested that most of the tryptophans were located in

TABLE I. Modified From Hardingham, Ewins, and Muir (10)

Effect of amino acid modification reactions on the interaction of monomer proteoglycans with hyaluronic acid

Reagent	mmolar	% Inhibition
acetic anhydride	5.3	9
	53.0	15
	530	100
2-methylmaleic anhydride	5.5	0
	55.0	67
	550	100
butane-2,3-dione	5.8	0
	12.0	95
	58.0	100

relatively hydrophobic regions. Interaction with hyaluronic acid did not appreciably alter the spectrum, suggesting that the tryptophan residues do not form direct subsite interactions stabilizing the internal, presumbaly globular, portion of the HA-binding region protein. This suggestion was supported by the observations that the fluorescence spectra of denatured proteoglycans in 0.1% sodium dodecylsulfate and of reduced and alkylated proteoglycans were shifted toward longer wavelengths, indicative of a configurational change in which some of the tryptophans moved into more polar environments.

The above results suggest that the tertiary structure of the HA-binding region protein which is essential for effective interaction with hyaluronic acid requires the presence of certain unmodified lysine, arginine, and tryptophan residues. Heinegard and Hascall (35) have recently studied the effects of modifications of amino groups in the purified HA-binding region protein on subsequent interaction with hyaluronic acid in more detail. The profiles in Fig. 11a indicate the elution position on Sepharose 6B of the HA-binding region preparation alone (dashed line) and in the presence of a high-molecular-weight hyaluronic acid in a separate experiment (solid line). The result indicates that all of the molecules in the HA-binding region preparation are capable of interacting with hyaluronic acid. Dansylation of the HA-binding region protein in the presence of cycloheptaamylose-dansyl (36) resulted in 80–100% loss of binding activity in several experiments (Fig. 11b). On the other hand, ^{3}H-acetylation of the HA-binding region protein with an effective concentration of about 5 mM acetic anhydride gave a loss of interaction of only about 20% (Fig. 11c), a result which is consistent with the observations by Hardingham et al. (10) discussed above (Table I). When the fraction of the ^{3}H-acetylated sample which retained binding activity, (Fig. 11c) was reisolated by chromatography on Sephadex G 200 in a dissociative solvent to remove hyaluronic acid and then dansylated, only a small proportion (about 10%) of the molecules were inactivated (Fig. 11d). In a separate experiment, the HA-binding-region protein was ^{3}H-acetylated in the presence of hyaluronic acid and the interactive molecules then reisolated. In this case, the amount of radioactivity incorporated into the HA-binding-region protein was only about 50% of that incorporated into the molecules that were ^{3}H-acetylated in the absence of hyaluronic acid (Fig. 11c) which suggests that many reactive groups, primarily the ϵ-amino groups of lysine residues, are partially protected from acetylation when bound to hyaluronic acid.

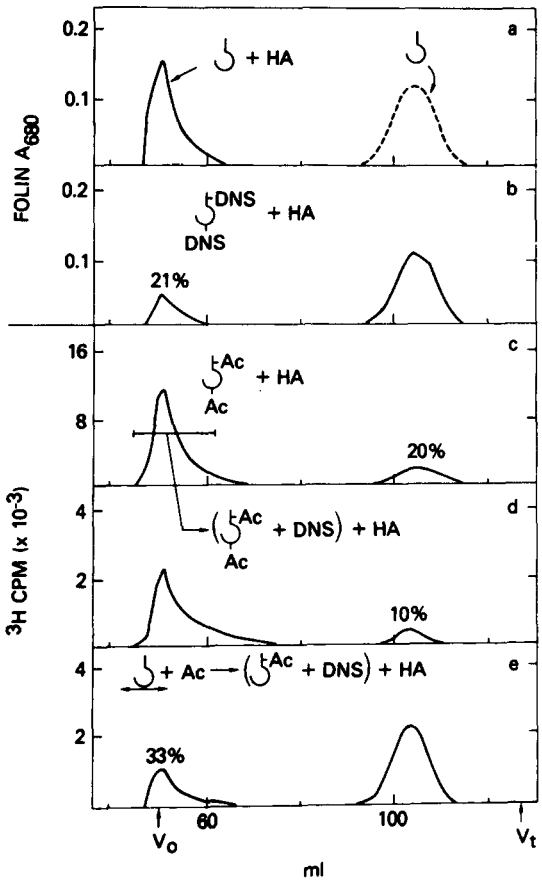


Fig. 11. Elution profiles on Sepharose 6B of aliquots of the HA-binding protein preparation which have been modified in various ways as indicated in the figure and text. The dashed line in (a) indicates the elution profile of the HA-binding region preparation alone in a separate experiment. The symbols "Ac" and "DNS" refer to acetylation and dansylation of reactive sties, respectively. The double headed arrow in (e) schematically represents a molecule of hyaluronic acid.

This was substantiated when it was shown that most of these interactive molecules could be inactivated by subsequent dansylation (Fig. 11e). These results suggest that there are lysine residues near the HA-binding site which are partially protected from substitution when hyaluronic acid is present, which can be substituted with acetyl groups without interfering with the interaction with hyaluronic acid, and which will prevent interaction with hyaluronic acid if substituted with the bulkier dansyl group.

A separate experiment was designed to see if portions of the polypeptide which are close to the HA-binding site could be selectively labeled. A sample of the HA-binding region protein was ^{3}H-dansylated in the presence of hyaluronic acid. Then, the hyaluronic acid was removed, and the sample was dansylated with unlabeled reagent. A separate sample was treated in the reverse order, dansylating first with unlabeled and then with ^{3}H-labeled reagent using the same protocol (Fig. 12). After purification, the 2 samples were treated with trypsin and the larger peptides, those excluded from Sephadex G-25, were subsequently fractionated on Sephadex G-50 (Fig. 12). While the elution profiles

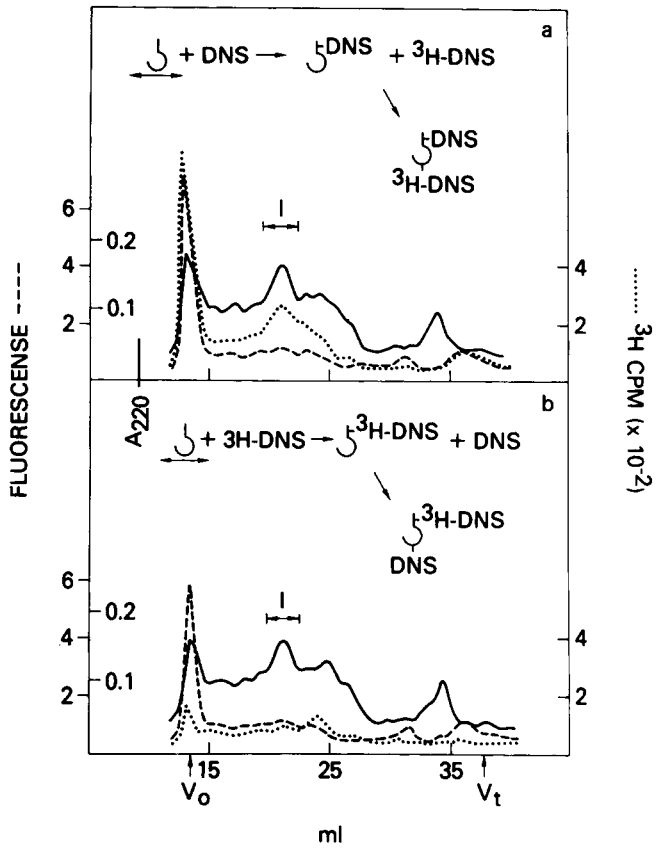


Fig. 12. Sephadex G-50 elution profiles of tryptic peptides that were isolated from dansylated HA-binding region protein samples prepared as indicated schematically in the figures. See text for details.

of absorbance at 280 nm and of fluorescence were essentially the same for each sample, the profiles of radioactivity differed. An included peak, indicated by I in Fig. 12, contained much more radioactivity when the ^{3}H-dansylation step was done after removing hyaluronic acid. Portions of this peptide(s) then, may be located in or near the HA-binding site. Similar experimental strategies should, in the future, provide many more details about the primary, secondary, and tertiary structure of the HA-binding region protein and how these provide an interaction site with hyaluronic acid. Indeed, it is fascinating to observe that proteoglycan research has reached a crossroads where many exciting new developments will depend more upon the skillful application of the research tools of protein chemistry rather than upon the more traditional methods of polysaccharide chemistry which have been critical for progress in the past.

V. THE EFFECT OF HYALURONIC ACID ON GLYCOSAMINOGLYCAN SYNTHESIS BY CHONDROCYTES

Hyaluronic acid appears to be critically involved in the process of development and differentiation of such tissues as chick cornea (37), chick vertebral column (38), and

regenerating newt limb (39). (For a recent review, see Ref. 40.) In each case, there is a correlation first between cell migration and the synthesis and accumulation of hyaluronic acid, and second between the subsequent enzymatic degradation of hyaluronic acid by newly synthesized hyaluronidases, the cessation of cell migration, and initiation of cell differentiation. This is a particularly pronounced effect for the differentiation of cartilaginous tissues. Toole and his collaborators tested the effects of hyaluronic acid on differentiation of stage 26 chick embryo somite cells (41). When these cells are dispersed by mild trypsin digestion and cultured on petri dishes at high initial plating density, they normally attach, divide, and undergo chondrogenesis to form nodules, in which mounds of chondrocytes are interspersed in a typical cartilaginous extracellular matrix. The presence of exogenous hyaluronic acid in the medium at concentrations from 1 ng to 500 μg detectably inhibited cell aggregation and the formation of nodules. The inhibition was not observed with a variety of other biological polyanions, including the chondroitin sulfates, heparin, and nucleic acids (40). HA-oligosaccharides, primarily tetrasaccharides, recovered from testicular hyaluronidase digests of hyaluronic acid, were also effective inhibitors of nodule formation. Monosaccharides, on the other hand had no effect (40). The treatment with hyaluronic acid did not cause any detectable changes in cell survival or proliferation, nor in the synthesis of either type I or type II collagen; [^{35}S] sulfate incorporation into polysaccharide, however, was inhibited.

Solursh et al. (42) subsequently studied the effect of hyaluronic acid on fully differentiated chick embryo chondrocytes grown in culture. The cultures were tested for glycosaminoglycan synthesis in the presence or absence of hyaluronic acid in a medium without serum. This was necessary since the serum used normally contained appreciable amounts of hyaluronic acid. At a concentration of hyaluronic acid of 200 μg/ml, [^{35}S] sulfate incorporation was inhibited by 50% over 6–24-h incubation times. The effect was also observed with HA-oligomers, although no data regarding their size distribution was provided. No differences between control cultures and cultures treated with hyaluronic acid were observed for leucine or thymidine incorporation into macromolecules, for cellular uptake of sulfate, for collagen synthesis, or for matrix turnover. The inhibition seemed to be associated primarily with the accumulation of proteoglycans associated with the cell layer matrix. However, the chondrocytes, derived in this case from clones of differentiated cells, were able to form nodules in the presence of hyaluronic acid, although there was appreciably less extracellular matrix around the cells. Again, other polyanions were incapable of eliciting this effect.

Wiebkin et al. (43) at the same time described similar experiments for suspensions of chondrocytes derived from trypsin/collagenase digests of adult pig laryngeal cartilage. Again, as little as 0.005 μg hyaluronic acid/ml in serum-free medium was capable of significantly decreasing [^{35}S] sulfate incorporation into glycosaminoglycans by cells which had been grown in culture for several days. The inhibitory effect of hyaluronic acid was not blocked by treating the cells with chondroitinase, but was blocked if the cells were treated with trypsin. The cells recovered their ability to respond to hyaluronic acid after 6 h or longer postincubation without trypsin. Hyaluronic acid, labeled with [^{14}C] acetate, was taken up at the surfaces of the cells when added to the chondrocytes and could subsequently be released by mild trypsin digestion. Hyaluronic acid samples which were first interacted with saturating amounts of proteoglycans did not inhibit glycosaminoglycan synthesis by the cells. When HA-oligomers from testicular hyaluronidase digests were fractionated on Sephadex G-25, the included fraction, which would contain primarily tetrasaccharides and hexasaccharides, did not inhibit chondrocyte

synthesis of ^{35}S-labeled glycosaminoglycans, but the excluded fraction, with longer HA-oligomers, did. Finally, other cell types which synthesize hyaluronic acid, fibroblasts and synovial cells, did not alter ^{35}S-labeled glycosaminoglycan synthesis when hyaluronic acid was added to cultures under similar experimental conditions.

More recently Handley and Lowther (44) reported experiments in which the effect of hyaluronic acid on glycosaminoglycan synthesis by chick embryo chondrocytes was investigated in the presence of a β-xyloside. These compounds, as well as xylose (45), act as exogenous acceptors for the synthesis of glycosaminoglycan chains, such as chondroitin sulfate (46–48), which are normally linked to the protein core of proteoglycans by a glycosidic bond between serine hydroxyls and xylose moieties (49). The cellular biosynthetic apparatus for glycosaminoglycan synthesis in chondrocytes diverts a large proportion of synthetic activity from the endogenous core protein to this exogenous substrate. In their experiments, Handley and Lowther (44) showed that total biosynthesis of ^{35}S-labeled glycosaminoglycans in the presence of the β-xyloside was the same independent of whether hyaluronic acid was present or not. It was suggested, then that the inhibition of synthesis in the presence of hyaluronic acid without added β-xyloside indicates that the specific effect of the hyaluronic acid is to initiate a series of intracellular events through intracellular effectors which inhibit either the synthesis of the normal proteoglycan core protein acceptor or the activity of the xylosyl transferase which is necessary to initiate the synthesis of each chondroitin sulfate chain on the protein.
glycans by chondrocytes and, therefore, may be an extracellular regulator of cellular processes involved in proteoglycan synthesis. b) Chondrocytes, unlike other connective tissue cells, probably contain cell surface receptors specific for the interaction with hyaluronic acid. c) The specificity of the receptors for hyaluronic acid may have similarities with those of the matrix molecules which interact specifically with hyaluronic acid, namely the HA-binding region protein of proteoglycans and the link proteins. d) Such an interaction between an extracellular matrix component and the chondrocyte may provide control mechanisms for elaborating, maintaining, or modifying cartilage matrices.

The experiments discussed in this presentation suggest that many facets of the structure and function as well as of the differentiation and development of cartilage tissues involve specific interactions between proteins and hyaluronic acid. Undoubtedly, many new insights about this connective tissue will be uncovered as investigators discover more details about these interactions.

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