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The interactions of cartilage proteoglycans with collagens are determined by their structures

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Abstract — In the present work, the interaction of aggrecan, decorin and biglycan isolated from pig laryngeal cartilage and of the three squid cartilage proteoglycans with collagen type I and II was studied. The interaction was examined under conditions allowing the formation of collagen fibrils. It was found that biglycan interacted strongly with collagen type II and not with type I and the interaction seemed to proceed exclusively through its core proteins. Decorin interacted with collagen type I but not with type II. Aggrecan interacted very poorly with both collagen types. The two squid proteoglycans of large size, D1D1A and D1D2, interacted only with collagen type I through both glycosaminoglycans and core proteins. The third squid proteoglycan of small size, D1D1B, interacted poorly only with collagen type I. The results suggested that the interactions of cartilage proteoglycans with collagen were mainly due to the primary structure of both molecules, and would contribute to the maintenance of the integrity of the tissue. The biochemical significance of these interactions might be more critical in aged vertebrate cartilage, where loss of aggrecan and increase of the small proteoglycans was observed, a large proportion of which is found in the extracellular matrix free of glycosaminoglycan chains.

collagen / proteoglycan interactions / core protein / glycosaminoglycans / vertebrates / invertebrates

1. Introduction

Collagen and proteoglycans are the main macromolecules of cartilage. The main collagen molecule of cartilage belongs to type II, the triple helix of which is formed by three identical α_1(II) chains [1]. Minor amounts of other collagen molecules are also identified in cartilage [2–4]. The main proteoglycan population of cartilage is aggrecan, the high molecular mass proteoglycan, which possesses a core protein substituted with over 100 chondroitin sulphate, 50 keratan sulphate and 50 oligosaccharide side chains [5–7]. Two other proteoglycan molecules, of low molecular mass and with a core protein of similar size and rich in leucine, are also identified in cartilage, but in minor amounts, namely biglycan and decorin, the core protein of which is substituted with two and one chondroitin sulphate chains, respectively [7–9]. In many cases and especially in human cartilage, most of biglycan and decorin molecules are non-substituted with glycosaminoglycan chains [10]. Some other proteoglycan molecules are also identified in cartilage, such as versican, lumican, epiphycan and fibromodulin, but in very minor amounts [11–14] and mainly as free proteins.

Invertebrate cartilage seems to be different from vertebrate cartilage. Results from studies of squid cranial cartilage, a well-characterized invertebrate cartilage, have shown that the tissue contains an overglycosylated type I collagen [15] and proteoglycans that do not belong to any proteoglycan molecule of vertebrate cartilage. Three different proteoglycan populations are identified in squid cranial cartilage, the core protein of which is substituted with two to five chains of oversulphated chondroitin of very high molecular mass [16]. The two high molecular mass populations possess the ability to self-aggregate [17], mainly through their core proteins.

Connective tissue collagen fibrils develop and function in intimate contact with proteoglycans and there has long been an interest in the possibility of interactions of a specific nature between the two of them. These interactions might influence or even control two of the most important events of connective tissue history: the increase in fibril size and the calcification of collagen fibrils. Many attempts have been made to demonstrate these interactions in vivo, but most of the evidence was derived from in vitro experiments using the more significant connective tissue collagen molecule, type I [18]. From such experiments it has been observed that collagen fibrils interact with the small interstitial proteoglycans, biglycan and decorin, via their glycosaminoglycan side chains. The core proteins interact weakly [19, 20] and the interaction depends on their structure [21–23]. The interaction of type I collagen with decorin is much stronger than that of biglycan, as the latter is highly affected by increased ionic strength [19].

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Not enough evidence is presented yet for the interactions of collagen type II with the various types of proteoglycans present in cartilage. In addition, there are no references suggesting the possible in vivo or in vitro interactions of proteoglycans and collagen of squid cranial cartilage.

The aim of the present work was the in vitro examination of the interactions of the main vertebrate and invertebrate cartilage collagens with the various proteoglycan populations isolated from both tissues. The methodology applied involved the formation of collagen fibrils in vitro and the examination of the effect of proteoglycans on it and the direct determination of the interactions by a solid phase assay. The resistance of these interactions in the presence of various salts and buffers was also studied. In addition, the interactions were examined after specific enzymic degradation of the interactors to reveal their regions responsible for the interactions.

2. Materials and methods

2.1. Materials

The types of collagen used throughout the study were type I from bovine achille's tendon (Sigma), type II from bovine nasal cartilage (Sigma), type I isolated from squid cranial cartilage [15] and type II isolated from pig laryngeal cartilage [24]. The proteoglycans used were aggrecan, biglycan and decorin, all isolated from pig laryngeal cartilage by a battery of methods and contained chondroitin sulphate chains [25–27] and the three squid proteoglycans termed D1D1A, D1D1B and D1D2 [16]. Polyclonal antibodies against aggrecan and squid proteoglycans were prepared as described previously [28]. Polyclonal antibodies against decorin and biglycan were kindly provided by Professor D. Heinegård (University of Lund, Sweden). Chondroitinase ABC and collagenase from *Clostridium histolyticum* (type VII) were obtained from Sigma Chemical Co.

All chemicals used throughout the study were of the best available grade.

2.2. Collagen fibril formation

The interactions between collagen and proteoglycans were studied by the inhibition of collagen fibril formation in vitro [29]. Collagen type I or type II was dissolved in 0.14 M NaCl–0.01 M sodium phosphate, pH 7.3 (PBS), at a concentration of 100 μg/mL and incubated for the next 15 h at 37 °C to induce fibril formation. The absorbance of the solutions at 400 nm, due to the formation of the fibrils, was recorded. The experiments were repeated in the presence of different concentrations (10–200 μg/mL) of the various proteoglycan populations listed above and the absorbance of the solutions at 400 nm was also recorded for the next 15 h. Any alterations in the absorbance measured were due to the interaction of the proteoglycans with collagen, which did not permit fibril formation.

2.3. Solid phase assay

The interaction of proteoglycans with collagen was also studied by solid phase assay, where the various proteoglycans were left to interact with immobilized collagen fibrils [28]. Collagen type I or type II was added (100 μL) at various concentrations (1–10 μg/mL) to microplate wells in either 0.02 M $NaHCO_3$, pH 9.6, or PBS and incubated at 23 °C for 20 h for coating the wells. Bovine serum albumin (3% in PBS) was used to block any free sites of the plate wells, after incubation for 1 h at 37 °C. Increased concentrations of the different proteoglycan populations (60 ng/mL–20 μg/mL) in various buffers were then added and allowed to interact with collagen for 1 h at 37 °C. The proteoglycans bound in each well were reacted with the respective antiserum diluted 1:5000 in PBS after incubation of the plate wells for 1 h at 37 °C. The plate wells were finally incubated for 1 h at 37 °C with a peroxidase-labeled second antibody diluted 1:10 000 in PBS and the amount of enzyme bound was quantitated using o-phenylenediamine (1 mg/mL in 0.05 M sodium citrate, pH 5.5, containing 0.01 M hydrogen peroxide) as substrate.

2.4. Enzymatic digestions

Complete digestion of proteoglycans with chondroitinase ABC was performed in 0.1 M Tris-acetate, pH 7.3, at 37 °C for 18 h using 1 unit of enzyme per mg of substrate in 1 mL of enzyme. For partial digestion of proteoglycans with chondroitinase ABC, 0.1 unit of enzyme was used for various time periods. The digestions were terminated after heating the solutions at 100 °C for 5 min. Monitoring of the extent of the digestions was performed by measuring the absorption of the digests at 232 nm owed to the C-4 double bond of the uronic acid at the non-reducing end (E_{232}=5500 M^{-1} cm^{-1}). In all cases, the protein cores were separated from the oligosaccharides removed by the enzyme after gel filtration on Sepharose CL-4B columns and used in the subsequent experiments.

Digestion of proteoglycans with collagenase was performed in 0.1 M Tris-acetate, pH 7.3, using 300 units of enzyme per mg of proteoglycans and mL of buffer. The samples were incubated for various time periods at 37 °C for 18 h, and the digestions were terminated after heating the solutions at 100 °C for 5 min. The digests were directly used in the subsequent experiments.

3. Results

3.1. Interactions of intact proteoglycans with collagen

3.1.1. Inhibition of collagen fibril formation by proteoglycans

Collagen type I from the Achilles tendon or from squid cranial cartilage and type II from pig laryngeal cartilage

dissolved in PBS, pH 7.3, at a concentration of 100 μg/mL formed fibrils after incubation at 37 °C for about 12 h (*figure 1A, B*), after which the absorbance of the solutions at 400 nm remained constant. The experiment was repeated in the presence of the various proteoglycans studied. It was found that collagen type I fibril formation was inhibited by decorin and the two squid proteoglycan populations of high molecular mass, D1D1A and D1D2 (*figure 1A*). The results were the same independently of the tissue source of collagen. The effect of decorin, the

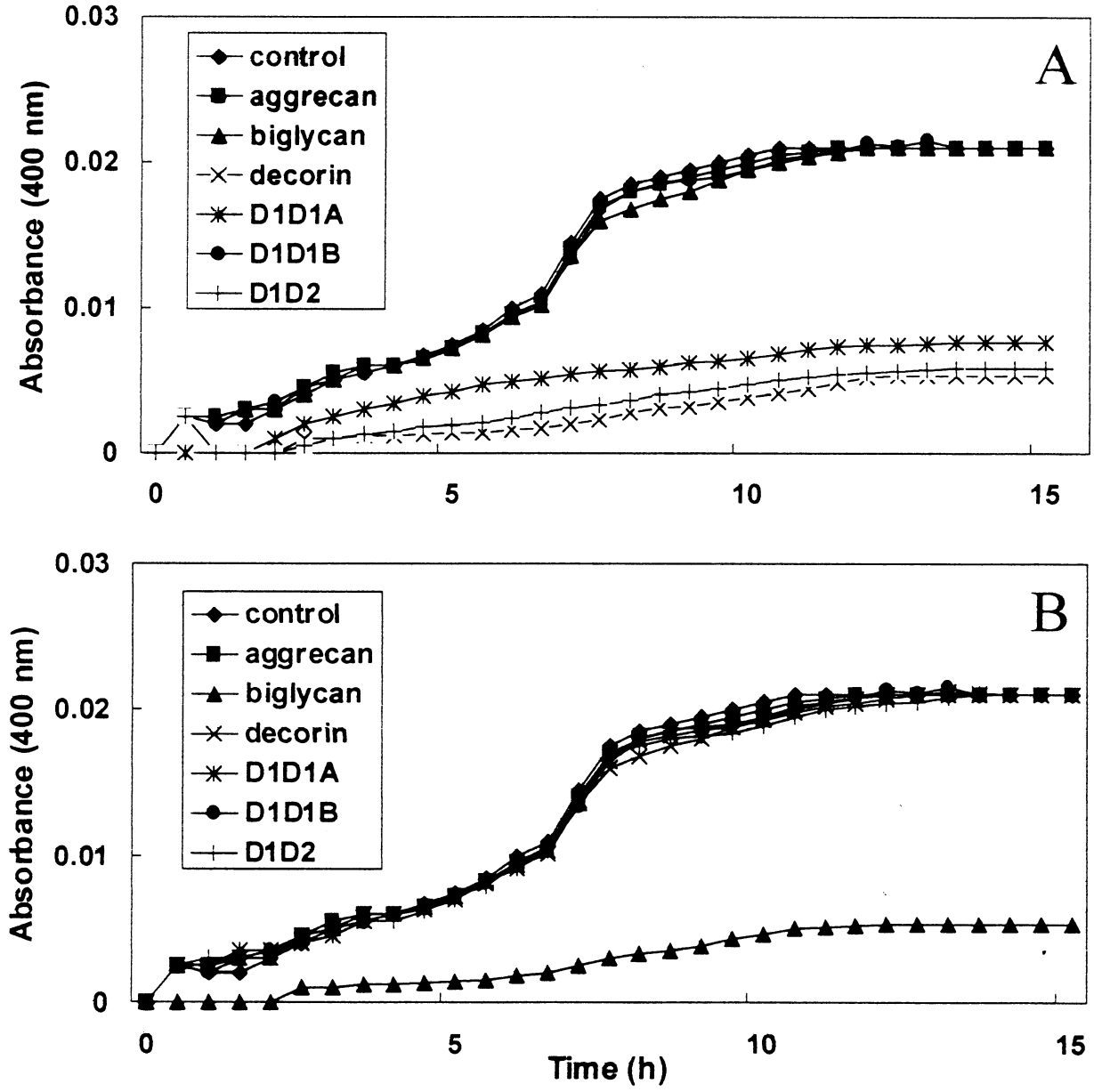

Figure 1. Inhibition of collagen fibril formation by proteoglycans. Collagen type I from squid canial cartilage (**A**) or type II from pig laryngeal cartilage (**B**) was incubated at 37 °C and at a concentration of 100 μg/mL in PBS, alone (◆) or in the presence of either of aggrecan (■), biglycan (▲), decorin (x), D1D1A (*), D1D1B (●) and D1D2 (+) at concentration of 10 μg/mL. The absorbance at 400 nm due to collagen fibril formation was recorded and plotted versus time.

interactions of which with collagen are extensively studied [19–23, 30–33], seemed to be the strongest. On the other hand, collagen type II fibril formation was inhibited only by biglycan (*figure 1B*). Neither decorin nor any other proteoglycan was found to be able to inhibit collagen type II fibril formation under the conditions examined.

3.1.2. Solid phase assay

Collagen type I or type II dissolved in either neutral or alkaline buffer was added to microplate wells and left overnight for coating the wells. The immobilised collagen was subjected to interaction with the various proteoglycans used in the present study in a wide range of concentrations and their amounts bound onto collagen were quantitated immunochemically. The results obtained were in accordance with the above. Collagen type I was found to interact strongly with decorin and the two large squid proteoglycans, D1D1A and D1D2, and to a very small extent with the squid proteoglycan population D1D1B (*figure 2A*). On the other hand, collagen type II interacted only with biglycan (*figure 2B*). The other proteoglycans did not interact at all with collagen type II in whatever buffer examined (not shown).

The effect of various agents on the interactions of proteoglycans with collagen was then examined. It was found that the interactions were affected by the phosphate concentration, the optimum being 5–20 mM (*figure 3A, B*). The nature of the buffer (Tris or phosphate) or the non-ionic detergent used (Tween or Nonidet) had a little effect on the interactions. On the other hand, sodium chloride decreased the interaction of squid proteoglycans with collagen type I, the optimum concentration being 0–100 mM (*figure 3C*), while it affected the interaction of biglycan with collagen type II over the concentration of 250 mM (not shown). This behaviour of squid proteoglycans suggested that the interaction was mainly electrostatic, most likely through CSE chains.

3.2. Interaction of collagen with proteoglycans fragments

The interactions of collagen with the proteoglycan core proteins were also examined. In the case of biglycan core protein, higher absorbance values were obtained compared to those obtained when intact biglycan was used, suggesting increased interaction either of the core protein with collagen type II from pig laryngeal cartilage or of the antibodies with the core protein (*figure 4A*). However, from Scatchard analysis of the data, the dissociation constants for both interactions were found to be similar, 1.4×10^{-8} for biglycan and 1.2×10^{-8} for core protein (*figure 4B*). This finding suggested that both biglycan and its core protein interacted similarly with collagen type II and the glycosaminoglycans did not contribute to the interaction. The latter was also verified by studying the

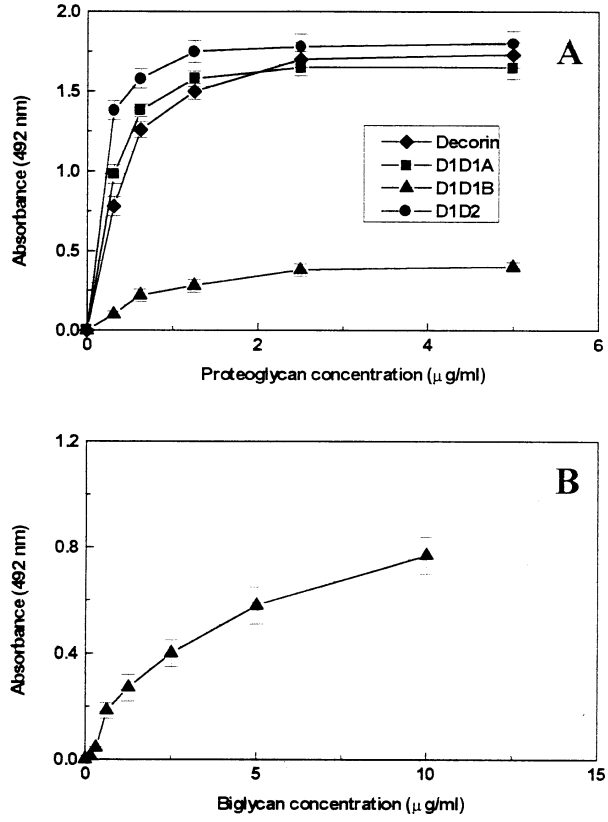

Figure 2. Interaction of collagen with proteoglycans using a microplate binding assay. **A.** Collagen type I (2 μg/mL) from squid cranial cartilage was immobilised on microplate wells and then interacted with various concentrations of either of decorin (◆), D1D1A (■), D1D1B (▲) and D1D2 (●). **B.** Collagen type II (2 μg/mL) from pig laryngeal cartilage was immobilised on microplate wells and then interacted with various concentrations of biglycan. In both cases the amount of proteoglycan bound was determined immunochemically using the respective antiserum, anti-rabbit IgG conjugated with peroxidase as second antibody and o-phenylenediamine as chromogen.

interaction of biglycan core protein with collagen in the presence of free glycosaminoglycan chains (not shown), which did not interfere at all with the interaction. The observed increased absorbance, when core protein interacted with collagen, seemed to be due to increased interaction of antibodies with the core protein of biglycan.

On the other hand, none of the squid proteoglycan core proteins interacted with collagen (*figure 5A*), in accordance with the hypothesis that this interaction was electrostatic and mediated through the CSE chains. Therefore, this interaction was investigated further, using partial degraded proteoglycans with chondroitinase ABC or collagenase (which has some activity on squid proteoglycans [28]). It was found that, in both cases, decreased interac

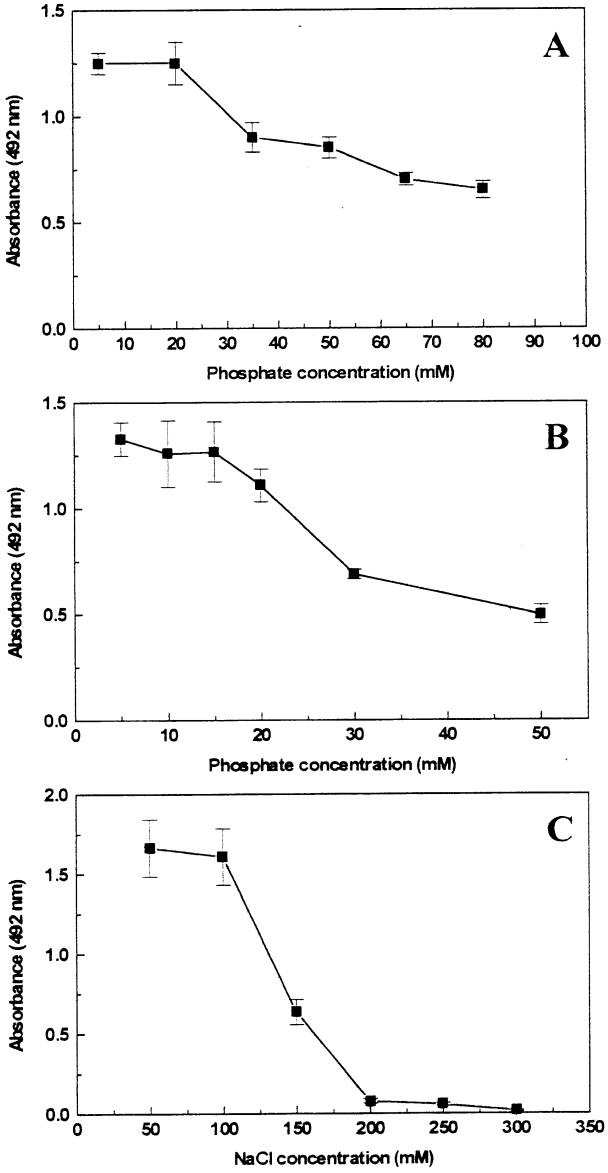

Figure 3. Effect of sodium phosphate (**A, B**) and sodium chloride (**C**) in the interaction of pig laryngeal collagen type II (**A**) and squid collagen type I (**B, C**) with biglycan (**A**) and D1D2 (**B, C**). The amount of proteoglycan bound on collagen was measured as described in the legend to *figure 2*.

tion was obtained (*figure 5B*), indicating that the presence of both intact core protein and CSE were indispensable for the interaction. These observations suggested that the synergistic effect of both protein and carbohydrate moities was necessary for interaction of squid proteoglycans with collagen.

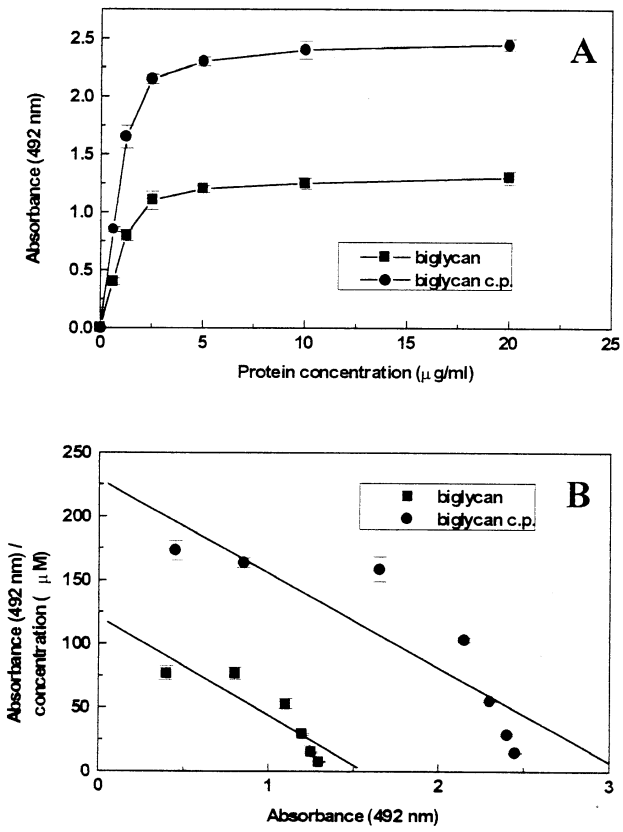

Figure 4. A. Interaction of biglycan (■) and its core protein (●) with immobilised pig laryngeal collagen type II. The amount of molecules bound on collagen was measured as described in the legend to *figure 2*. **B.** Scatchard plot analysis of the above measured amounts of biglycan or its core protein bound on collagen. The equation applied was: $\frac{\Delta A}{C} = \frac{\Delta A_{max}}{K_d} - \frac{\Delta A}{K_d}$ [29].

4. Discussion

The present work was undertaken to study the interactions of the main molecules of cartilage, collagen and proteoglycans, and to detect the parts of their structure involved in such interactions. It was performed by studying the effect of the proteoglycans in collagen fibril formation in vitro and by measuring the amount of each proteoglycan or proteoglycan fragment bound to immobilized collagen fibrils. Collagen type I and II from squid cranial and pig laryngeal cartilage, respectively, were used. For comparison, collagen type I from bovine Achilles tendon and type II from bovine nasal cartilage were included in the study. The proteoglycan molecules used were aggrecan, biglycan, decorin, from pig laryngeal cartilage and D1D1A, D1D1B and D1D2 from squid cranial cartilage. The interaction of collagen type I with

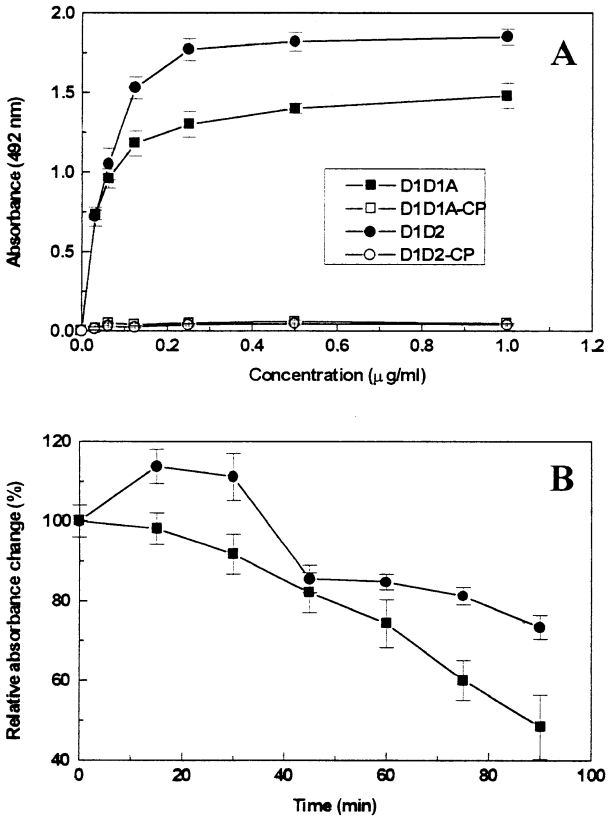

Figure 5. A. Interaction of D1D1A (■), D1D2 (●) and the respective core proteins (□, O) with immobilised squid collagen type I. **B.** Effect of partial enzymic degradation of squid proteoglycan D1D2 by chondroitinase ABC (●) and collagenase (■) in its interaction with collagen type I using the microplate binding assay. In both cases, the amount of molecules bound on collagen was measured as described in the legend to *figure 2*.

decorin was not extensively studied, since many investigations have been presented concerning this subject [19–23, 30–35].

It was found that the proteoglycans had a tendency to interact with the main type of collagen found in the respective cartilagenous tissue, but the proteoglycan molecules did not interact to the same extent. The results concerning the vertebrate cartilage suggested that aggrecan, the major proteoglycan of this tissue, did not interact with collagen type II. Previous investigations have shown an interaction of aggrecan with collagen [36], however, in the present investigation, at whatever salt concentration examined, no interaction of aggrecan with collagen was detected. From the two leucine rich proteoglycans examined, biglycan and decorin, only biglycan was found to be able to interact with collagen type II. The interaction seemed to be quite strong, since it survived in increased phosphate concentration. The interaction proceeded through the core protein of the proteoglycan, since similar dissociation constants were calculated for the interaction of collagen with either biglycan or its core protein, after Scatchard plot analysis of the results obtained. The chondroitin sulphate chains did not participate at all in this interaction (not shown). Squid proteoglycans did not interact with collagen type II obtained from mammalian cartilage.

Different results were obtained for the interaction of collagen type I with proteoglycans. Decorin from vertebrate cartilage and the high molecular mass proteoglycans from invertebrate cartilage were found to have the ability to interact with it. The interaction of decorin with collagen is known to proceed mainly via its core protein, the leucine rich repeats being directly involved in the interaction [21]. Squid proteoglycans seem to interact with collagen via both their core proteins and their glycosaminoglycan chains, since the degradation of each does not permit the detection of any interaction, although the antisera used could detect epitopes on both the core proteins and the oversulphated chondroitin chains [28]. To verify this observation, the interaction of collagen type I with partially degraded squid proteoglycans was examined. The proteoglycans were digested with either collagenase or chondroitinase ABC in a time-dependent manner, and the partially degraded molecules were left to interact with immobilised collagen. Collagenase can degrade squid proteoglycans to minor extent without affecting substantially their immunoreactivity [28]. However, the interaction of collagenase degraded squid proteoglycans with collaged was found to be decreased as compared to that of the intact proteoglycans. Similar results were also obtained for the interaction of chondroitinase ABC degraded proteoglycans with collagen. These results taken together suggested that the interaction of squid proteoglycans with collagen type I involved both protein and carbohydrate parts of the proteoglycans. In addition, the size of glycosaminoglycan chains seemed to affect the interaction, since the products obtained after degradation of the proteoglycans by chondroitinase ABC for up to 30 min showed increased interaction with collagen (*figure 5B*). It seemed that chondroitinase ABC acts initially on chains of rather large size by removing about 2–4% of the disaccharides from the outer part of the molecules, thus decreasing some steric hindrance due to different sized CSE. As the digestion proceeded, chondroitinase ABC degraded even more the chains (up to 15% of the disaccharides have been removed) and the proteoglycans lost little by little their capacity to interact with collagen with both their substructures. The interaction was not quite strong and it was abolished after increasing the concentration of NaCl to more than 150 mM and it was highly decreased by 50 mM phosphate. All these results suggested the ionic nature of the interaction of squid proteoglycans with collagen.

The source of collagen type I, i.e., vertebrate or invertebrate, did not affect the interactions examined. The main difference of these collagen molecules is the number of carbohydrate moities, which is greater in the invertebrate collagen [15]. The results of the present study suggested that the carbohydrate part of the collagens was not involved in the interaction with the various proteoglycans.

From the results of the present study it might be proposed that the various collagen molecules behaved differently in their interactions with the interstitial proteoglycans, most likely due to differences in the structure of the collagen molecules. These differences referred to either the amino acid sequence or to the different α chains forming each collagen molecule. The biochemical significance of the interactions concerning the biglycan might be more critical in aged cartilage, where increased concentrations of the small proteoglycans are present, a large proportion of which is found in extracellular matrix free of glycosaminoglycan chains [10, 37]. This interaction, together with the self-association properties of cartilage biglycan, would largely contribute to the integrity of the tissue [38]. Similarly, the interactions of squid proteoglycans with collagen type I would add to the self-aggregation of squid proteoglycans, thus contributing to the integrity of the cranium. In these in vivo interactions, the participation of other molecules, such as the leucine rich proteins of the extracellular matrix [39–41], which interact to some extent with collagen, can not be excluded.

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