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REGULAR ARTICLE

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A novel fibrocartilaginous *tendon* from an elasmobranch fish (*Rhinoptera bonasus*)

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Abstract Tendons of the jaw adductor muscles of a hard prey crushing stingray exhibit similar adaptations to compressive and shear loads as those seen in mammalian tendons. Ventral intermandibular tendon from the cownose ray, *Rhinoptera bonasus*, has a prominent fibrocartilaginous pad that lies between a fibrous region of the tendon and the mineralized tissue of the jaw. Histologically the pad is similar to the fibrocartilaginous meniscus of mammals, and these tissues also share some biochemical traits. Proteoglycan (PG) content in the fibrocartilaginous pad is nearly four times higher than in the linearly arrayed tendinous tissue. The predominant PGs appear to be an aggrecan-like molecule and a decorin-like molecule. The decorin-like molecule is quite small when compared to mammalian decorin (20–80 kDa vs. 100–200 kDa). This study is the first to document adaptations to compressive/shear loading in tendon from a cartilaginous fish, and the similarities to the mammalian condition argue for the early evolution of this reactive ability of tendinous tissue.

Keywords Tendon · Fibrocartilage · Proteoglycan · Glycosaminoglycan · *Rhinoptera bonasus* (Elasmobranchii)

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Introduction

Several closely related genera of myliobatid stingrays eat hard prey to the exclusion of all else. These fish, including the cownose ray, *Rhinoptera bonasus*, and the spotted eagle ray, *Aetobatus narinari*, crush clams, oysters and other bivalves and gastropods between thick tooth plates (Gudger 1914; Bigelow and Schroeder 1948). Their jaws are of 'trabecular cartilage', a variant of the prismatic cartilage found in most sharks and rays; in addition to the mineralized outer surface, hollow, calcified struts reinforce the jaw and support the tooth plates (Summers et al. 1998; Summers 2000). The massive jaw closing muscles and trabecular cartilage allow these rays to develop tremendous crushing force (Coles 1910).

The skeleton of sharks, skates and rays is unusual compared to other vertebrates in being composed entirely of several types of calcified cartilage. The spinal column is fully mineralized areolar cartilage, a form of mineralized cartilage characterized by a disorganized web-like mineralization that permeates the entire skeletal element. The rest of the endoskeleton is composed of prismatic cartilage—hyaline cartilage covered in a thin layer of tiny (<1 mm) mineralized tiles of heavily mineralized cartilage, much the way an orange peel surrounds the pulpy inner fruit (Ørvig 1951; Applegate 1967). Liem and Summers (1999) proposed that cartilage has a low pull-out strength, which is responsible for the scarcity of non-aponeurotic tendons in cartilaginous fishes. A narrow tendon serves to concentrate the stress of the associated muscle and poses a structural problem if inserted directly onto a small area of a cartilaginous skeletal element. While in the majority of sharks and rays the force of the muscles is delivered with broad tendinous insertions, it is not known whether the forces generated by the hard prey crushing stingrays have led to specializations in the muscles and tendons.

In dissecting the jaws of a cownose ray we came across a muscle-tendon complex that appears functionally analogous to the 'wrap around' tendons found in mammals (for review, see Vogel and Koob 1989). In addition to the

usual tensile load, these tendons are subjected to shear and compressive loads as they wrap around a skeletal element. In order to dissipate these loads without damage to the tendon they develop resilient, fibrocartilaginous regions. For example, the bovine deep digital flexor tendon runs across a pulley formed by the flexed metatarsal-phalangeal joint in the foot. The action of the flexor muscle loads the tendon in tension; however, where the tendon crosses the pulley it also experiences a compressive side load and a shearing load. In this region the tendon develops a fibrocartilaginous pad with biochemical composition and material properties very different from those regions of the tendon that only experience tensile loads (Koob and Vogel 1987; Koob 1989).

The evolutionary history of teeth and bone is relatively well understood (Smith and Hall 1990); however, we have little or no comparative data to determine the evolutionary path of soft skeletal tissues (Summers and Koob 2002). Basic questions of homology between cartilage, tendon, ligament and fibrocartilage among vertebrate and invertebrate groups remain to be answered (Hall 1983). We have examined connective tissue from a cartilaginous fish that appears to experience a load regimen similar to that of mammalian fibrocartilage. We present the gross anatomy, ultrastructural morphology and biochemical composition of the muscle-tendon complex and compare it to tendon and fibrocartilage of mammals.

Materials and methods

Histology

The ventral intermandibularis (VIM) tendon was dissected from a freshly killed cownose ray and preserved in 10% neutral buffered formalin for 48 h. The tissue was dehydrated in a series of ethanol washes, then bisected with a sharp razor blade along the long axis of the tendon. The tendon was embedded in Paraplast (Oxford Labware). Thin sections were cut on a Reichert microtome, mounted on glass slides and stained with Alcian blue/van Gieson at pH 2.5 (Humason 1972), and Verhoeff's elastin stain (Humason 1972). Sections were cut through the central 1 mm of the tendon and alternate sections were treated to the two staining protocols.

Protein and proteoglycan extraction and purification

Freshly minced tissue collected from six tendons from three adult individuals was extracted for at least 24 h at 4°C in 6 M guanidine-HCl, 0.1 M Na acetate, pH 6.0, containing the following protease inhibitors: 5 mM benzamidine, 10 mM ethylenediaminetetraacetic acid, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonylfluoride. Tissues were extracted at a ratio of 10 ml/g tissue. Extracts were centrifuged at 27,000×*g* for 30 min. The supernate was collected and dialyzed against 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5. Since the dialyzed extract was viscous, urea was added to a final concentration of 3.5 M. The extract was then applied to a 5-ml HiTrap Q column (Amersham Biosciences, Piscataway, NJ). The column was washed with 10 ml application buffer to remove unbound material. A 50-ml linear gradient from 0.1 M to 2.0 M NaCl was applied to the column. The column was finally washed with 25 ml of 3 M NaCl, 20 mM Tris-HCl, pH 7.5. Two-milliliter fractions were collected. The NaCl gradient was monitored by measuring the conductivity of each fraction. The relative amount of protein in each fraction was determined by measuring absorbance at 280 nm. For measurement of chondroitin sulfate content in each fraction, a 150-μl aliquot was digested with 0.02 units chondroitinase ABC (Seikagaku America, Inc., Ijamsville, MD) in 50 mM Na acetate, pH 7.5, and the resulting disaccharides were measured by capillary zone electrophoresis according to established methods (Al-Hakim and Linhardt 1990). SDS/PAGE analyses of the macromolecules in each fraction containing protein or chondroitin sulfate were performed by precipitating 150 μl of the fraction with ice-cold 95% ethanol and then redissolving the lyophilized precipitate in gel sample buffer containing 50 mM β-mercaptoethanol. Samples were electrophoresed on 4–20% linear gradient Tris-glycine gels (Invitrogen, Carlsbad, CA). Gels were stained with Coomassie brilliant blue for proteins and Alcian blue for proteoglycans.

Proteoglycan localization and characterization

Another VIM tendon was dissected from the jaws of an adult cownose ray, cleaned of muscle and rinsed with distilled water. The tendon was divided into three major sections from the origin in the adductor to the insertion on the upper jaw element. The tendon arising from the adductor was divided into three consecutive samples cut perpendicular to the long axis of the tendon, and the tendon that attached to the upper jaw was divided into six consecutive samples. Between these samples, the tendon that comprised the pad was bisected into halves that faced the jaw cartilages and the adductor muscle. Each half was further divided into three samples orthogonal to the bisected face and parallel to the length of the tendon. Material from the quadratomandibularis (QM) tendon and the intermandibular ligament (IM) of a stingray as well as the quadratomandibularis tendon of a bull shark (*Carcharhinus leucas*) was obtained from fresh specimens for comparative purposes.

Samples were frozen, lyophilized, weighed and digested with papain (Worthington Biochemical Co., Freehold, NJ) at 60°C overnight (0.12 unit papain/mg sample; 10 mg sample/ml papain buffer, 0.2 M Na acetate, 20 mM cysteine, 4 mM EDTA, pH 6.0). Papain digestion of the dried samples solubilized the tissue entirely and thereby liberated glycosaminoglycans (GAGs) for further analyses. After incubation, the samples were heated to 100°C for 5 min to inactivate the papain and then centrifuged for 5 min at 7,000×*g*. A 200-μl sample of the supernatant was removed from each sample, and the glycosaminoglycans were precipitated by addition of 1.8 ml of ice-cold 95% ethanol. The precipitate was collected by centrifugation (16,000×*g*×10 min) and washed with 95% ethanol. The precipitate was redissolved in 100 μl of chondroitinase buffer (50 mM Na acetate, pH 7.5) and divided into two 50-μl aliquots. One sample was digested with 0.2 unit of chondroitinase ABC, which depolymerizes chondroitin and dermatan sulfate, and the other was digested with 0.2 unit of chondroitinase ACII, which depolymerizes only chondroitin sulfate (chondroitinases from Seikagaku America, Inc.). Enzyme digestion of the samples was carried out by incubation at 37°C overnight. The samples were assayed for the resulting chondroitin sulfate and dermatan sulfate disaccharides by capillary zone electrophoresis according to established methods (Al-Hakim and Linhardt 1990). Concentrations of Δdi-6S and Δdi-4S chondroitin sulfate (CS) were determined in the ACII digests by comparison to the results of an ACII digest of chondroitin sulfate C at a concentration of 100 μg/ml. Concentrations of dermatan sulfate disaccharides were calculated by subtraction of the ACII results from ABC results.

Fractions containing Alcian blue staining material eluting between 1.1 and 1.7 M NaCl from the HiTrap Q column were combined, dialyzed against water and lyophilized. The sample was redissolved in 2 ml 0.8 M NaCl, 0.1 M Tris-HCl, pH 8.0, and applied to a HiPrep 16/60 Sephacryl S-100 Hi-Resolution size exclusion column (Amersham Biosciences). The column was eluted with the NaCl-Na-acetate buffer at a rate of 1 ml/min, and 4-ml fractions were collected. The GAG content in each fraction was

measured with 1, 9-dimethyl-methylene blue (DMMB) (Templeton 1988).

Fractions 10 and 11 containing large molecular mass proteoglycans from the Sephacryl S-100 column were combined, and four 150-µl aliquots were ethanol precipitated as described above to prepare samples for GAG analysis by glycosidase digestions. One aliquot was incubated in 0.05 M Na acetate, pH 7.0; an aliquot was incubated with 0.02 unit chondroitinase ACII in the same buffer to digest chondroitin sulfate; an aliquot was incubated with 0.02 unit chondroitinase ACII, 0.01 unit keratinases I and II to digest chondroitin and keratan sulfate; an aliquot was incubated with 0.02 unit chondroitinase ABC to digest chondroitin and dermatan sulfate; the incubation was performed for 16 h at 37°C.

Three 150-µl aliquots from each fraction 12, 13, 14 and 15 from the Sephacryl S-100 column were ethanol precipitated. One aliquot was incubated in the glycosidase buffer; one aliquot was incubated with 0.02 unit chondroitinase ACII; one aliquot was incubated with 0.2 unit chondroitinase ABC. The incubation was performed as above. Following glycosidase digestion, the samples were diluted with an equal volume of SDS/PAGE gel sample buffer (×2 concentrated) and electrophoresed on 4–20% linear gradient Tris-glycine gels.

Results

The *adductor mandibularis medialis* muscle of the cownose ray, *Rhinoptera bonasus*, originates on the ventral surface of the Meckel's cartilage (lower jaw). The origin is large, extending from 8 mm lateral to the midline to 10 mm from the lateralmost extent of the cartilage. The muscle has a well-defined central tendon, and as the muscle extends laterally and wraps around the jaw joint it becomes more tendinous (Fig. 1A). The muscle has graded completely into tendon as it passes the posterior edge of the palatoquadrate, and this tendon inserts broadly across the anterior surface of the upper jaw. The tendon inserts at an extremely oblique angle to the surface of the upper jaw, lying flat across the jaw for the entirety of the insertion. Medial to the tendinous region is a fibrocartilaginous pad lying primarily between the tendon and the medial edge of Meckel's cartilage (Fig. 1B).

Histological analysis showed that the pad and tendon can be divided into three regions (Fig. 2A). The lateralmost region is typical vertebrate tendon. Polarized light and conventional microscopy showed densely packed fiber bundles, linearly arrayed along the direction of the tendon with a minimal amount of Alcian blue staining material (Fig. 2B). The second region, just medial to the tendon, appeared fibrocartilaginous. Dense fibrous bundles were found interspersed with Alcian blue stained tissue (Fig. 2C). Polarized light revealed that the fibrous bundles are primarily aligned with the tendon, but in some regions the fibers are slightly wavy. The innermost, medial region also appeared fibrocartilaginous and stained intensely with Alcian blue; however, the fibers were dispersed rather than arranged in bundles, and polarized light showed that there is no preferred orientation (Fig. 2D, G). The wavy fiber bundles that lie closest to this region appeared to have a greater wave amplitude than more lateral bundles. Along the medial margin of the pad, lying against the jaw cartilage, dense fibrous bundles

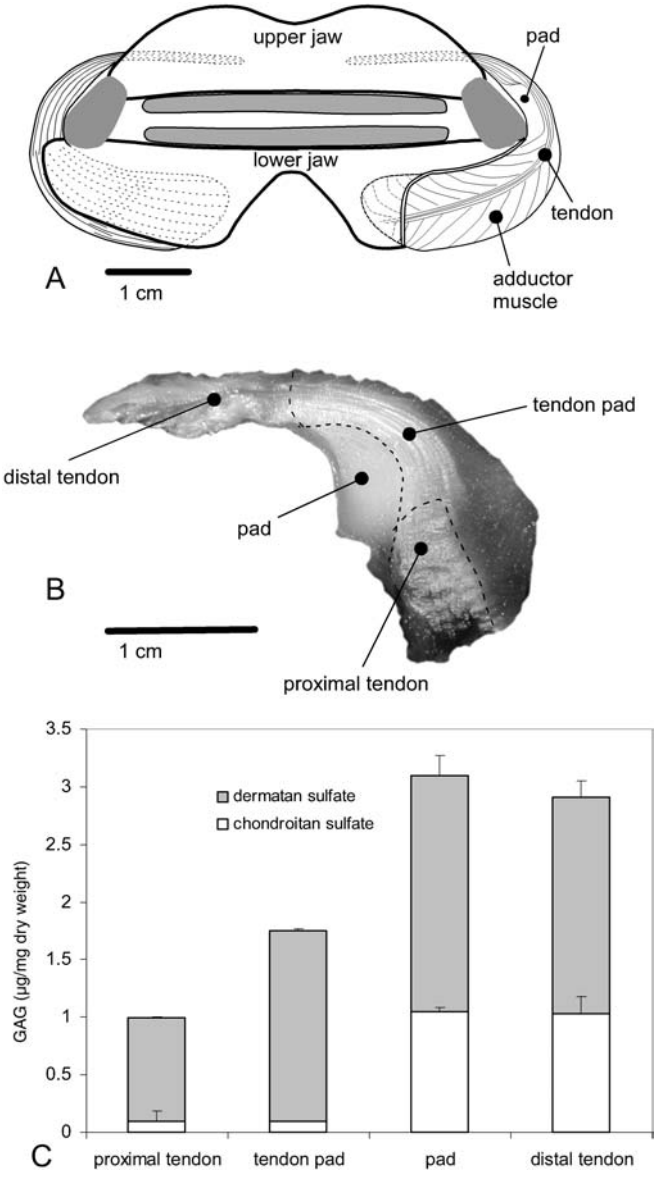

Fig. 1 **A** Ventral view of the jaws of the cownose ray, *Rhinoptera bonasus*, dissected to show the position of the *adductor mandibularis medialis* muscle and the associated tendon. The left side is a superficial dissection to expose the outer surface of the jaw and muscle. On the right side the ventral margin of the lower jaw (Meckel's cartilage) has been cut away and the muscle sectioned along its long axis to reveal the central tendon. The upper and lower jaws are connected by strong, short ligaments shown in *dark gray*. **B** A photograph of a section of the tendon showing the fibrocartilaginous pad and the linear fibers of the tendon lateral to it. The orientation of the tendon is approximately the same as in the right side of **A**. **C** The glycosaminoglycan content of four regions of the tendon subdivided into chondroitin sulfate and dermatan sulfate content

of collagen running perpendicular to the direction of the tendon were noted.

Capillary electrophoresis demonstrated that the glycosaminoglycan (GAG) content of different regions of the tendon/pad complex varied between 1% and 3% of dry weight (Fig. 1C). The GAG content of the other tendons

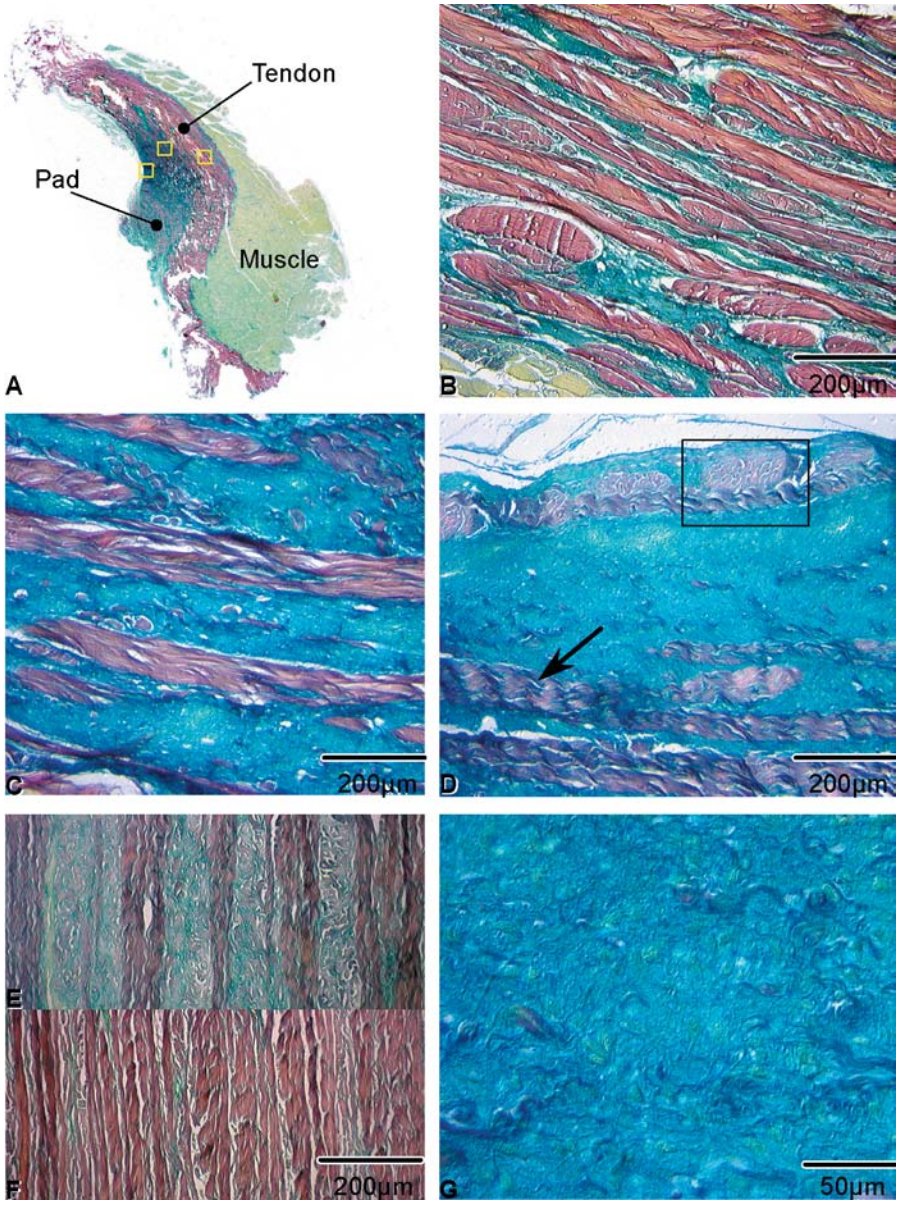

Fig. 2 **A** Thin section through the tendon and fibrocartilaginous pad of the *adductor mandibularis medialis* muscle from a cownose ray, *Rhinoptera bonasus*, stained with Alcian blue/van Gieson. The positions of sections shown in **B, C** and **D** are indicated by the *yellow rectangles*. The organized fibers of the linear part of the tendon stain red, and the fibrocartilaginous pad stains blue-green. **B** The fibrous, linearly arrayed fibers of the tendon. **C** Between the linear fibers of the tendon and the medial cartilaginous pad there are regions of organized fibers interleaved with cartilaginous regions. **D** The most medial portion of the cartilaginous pad shows few fibers aligned with the tendon. The *arrow* indicates fiber bundles with distinct waves at the edge of the cartilaginous pad. The *black box* illustrates that on the most medial edge of the pad there are bundles of fibers aligned perpendicularly to the fibers of the tendon. **E, F** Thin section of calf meniscus showing a region of fiber bundles and cartilage **(E)** and a more fibrous region **(F)**. **G** A polarized light micrograph of the cartilaginous pad showing that there is no preferred orientation to the embedded collagen fibers

Table 1 The glycosaminoglycan content of tendon, regions of specialized tendon, and ligament in elasmobranchs

Class	Species	Tendon	*N*	CSZ[a]	DS[b]	Total[c]	DS/CS	Comp./tens.
Chondrichthyes	Stingray	VIM[d] proximal	3	0.093±0.09	0.90±0.008	0.99	9.7	–
Chondrichthyes	Stingray	VIM tendon pad	3	0.094±0.005	1.6±0.01	1.75	17.0	–
Chondrichthyes	Stingray	VIM pad	3	1.04±0.03	2.05±0.1	3.09	1.9	3.43[e]
Chondrichthyes	Stingray	VIM distal	6	1.02±0.1	1.89±0.1	2.91	1.85	–
Chondrichthyes	Stingray	QM[f]	7	0.49±0.06	1.58±0.09	2.06	3.2	–
Chondrichthyes	Stingray	IM[g] ligament	6	4.93±0.5	10.2±0.5	15.1	2.06	–
Chondrichthyes	Shark	QM	8	0.41±0.08	0.60±0.04	1.01	1.46	–
Mammalia	Rabbit[h]	DDF[i] tension	–	–	–	0.13	–	–
Mammalia	Rabbit	DDF comp.	–	–	–	1.91	–	14.7
Mammalia	Dog[j]	DDF tension	–	–	–	0.26	–	–
Mammalia	Dog	DDF comp.	–	–	–	3.82	–	14.7
Mammalia	Bovid[k]	DDF tension	–	–	–	0.45	–	–
Mammalia	Bovid	DDF comp.	–	–	–	4.45	–	9.8

[a] µg/mg dry weight chondroitin sulfate ± standard error [b] µg/mg dry weight dermatan sulfate ± standard error
[c] µg/mg dry weight CS+DS [d] Ventral intermandibular [e] Pad/proximal tendon [f] Quadratomandibular [g] Intermandibular
[h] Gillard et al. (1977) [i] Deep digital flexor [j] Okuda et al. (1987) [k] Vogel and Koob (1989)

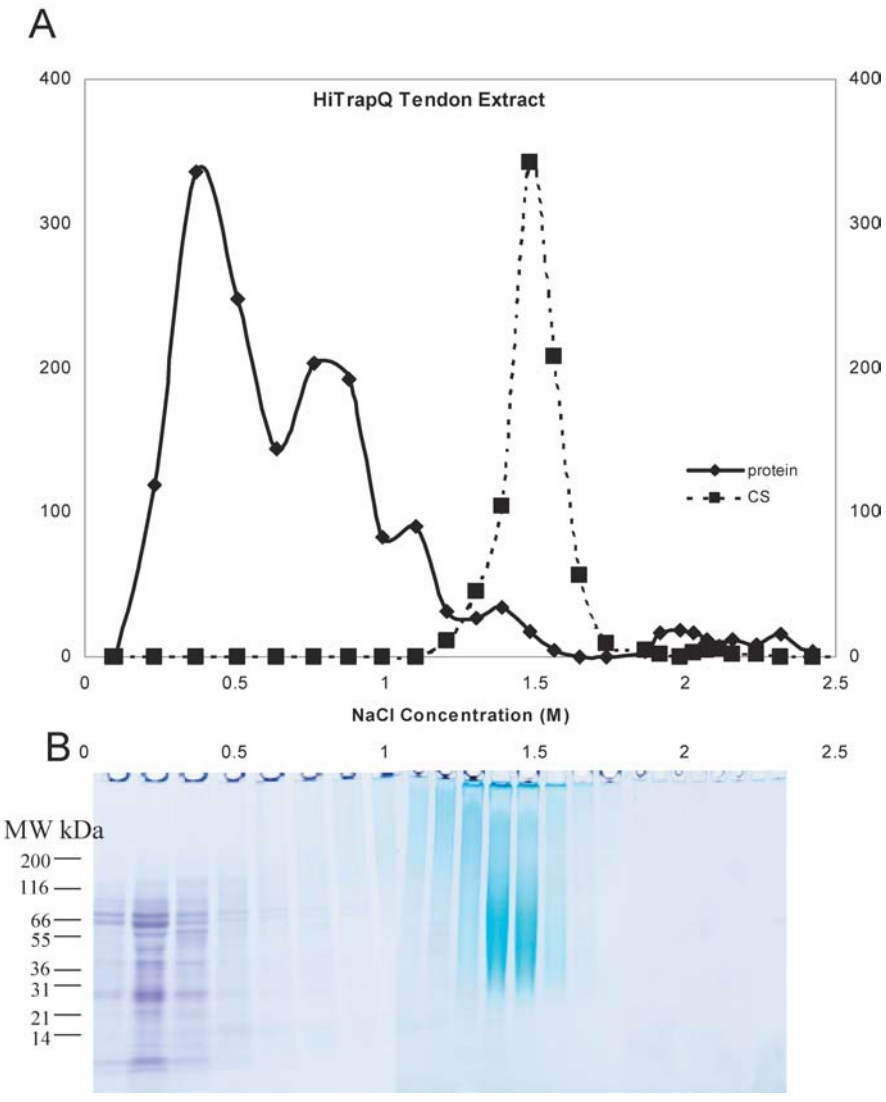

Fig. 3 **A** HiTrap Q ion exchange chromatogram of extracts from the ventral intermandibularis tendon of the cownose ray (*Rhinoptera bonasus*). The NaCl concentration in each fraction was measured by conductivity. The relative protein content in each fraction was measured by absorbance at 280 nm (*solid line*) and shows that most of the protein elutes from the column at low counterion concentration indicating a low charge to mass ratio. The chondroitin sulfate content in each fraction (*dashed line*) was measured by chondroitinase ABC digestion of an aliquot and quantitation of the resulting chondroitin sulfate disaccharides by capillary zone electrophoresis; elution at a relatively high counterion concentration indicates a high charge to mass ration. **B** SDS/PAGE analysis of macromolecules in each fraction on 4–20% linear gradient Tris-glycine gels

(QM from stingray and shark) was similar to that of the VIM tendon. GAG content of the intermandibular ligament was five times higher than in the tendons (~15%) (Table 1). Ion-exchange chromatography of the pooled extract of fibrocartilaginous pad and tendon revealed several proteins with low charge density (Fig. 3). Alcian blue stained proteoglycans (PGs) eluted between 1.0 and 1.75 M NaCl and consisted of large molecules greater than 500 kDa barely entering the gel as well as a broad diffuse band of smaller molecules.

Separation of the PG-rich fractions by size exclusion chromatography showed that the larger PGs were larger than 200 kDa while the smaller PGs ranged from 20 to 80 kDa (Fig. 4). Fractions 10 and 11 contained most of the larger PG, though fraction 11 also had significant amounts of the smaller PG. Chondroitinase ACII and ABC digestion of the large PGs in fractions 10 and 11 resulted in the appearance of Alcian blue staining material with a molecular mass between 200 and 400 kDa (Fig. 5A). When keratanases I and II were included in the incubation with chondroitinase ACII, the Alcian staining material was eliminated. These results indicate that the large molecular weight proteoglycan contains chondroitin and keratan sulfate. Digestion of the small proteoglycans in fractions 12–15 with chondroitinase ACII resulted in the loss of Alcian staining bands between 20 and 80 kDa and the appearance of low molecular weight Alcian staining material (Fig. 5C). Chondroitinase ABC eliminated all Alcian staining material and produced a Coomassie staining band migrating with an apparent molecular mass of 25 kDa (Fig. 5D, lanes 12, 13). These results indicate that the predominant small molecular weight proteoglycan contains chondroitin and dermatan sulfate (Fig. 5).

Discussion

Although tendons are rare in the head region of cartilaginous fishes, they do occur (*contra* Liem and Summers 1999). Furthermore, the *adductor mandibularis medialis* tendon bears striking morphological and ultrastructural

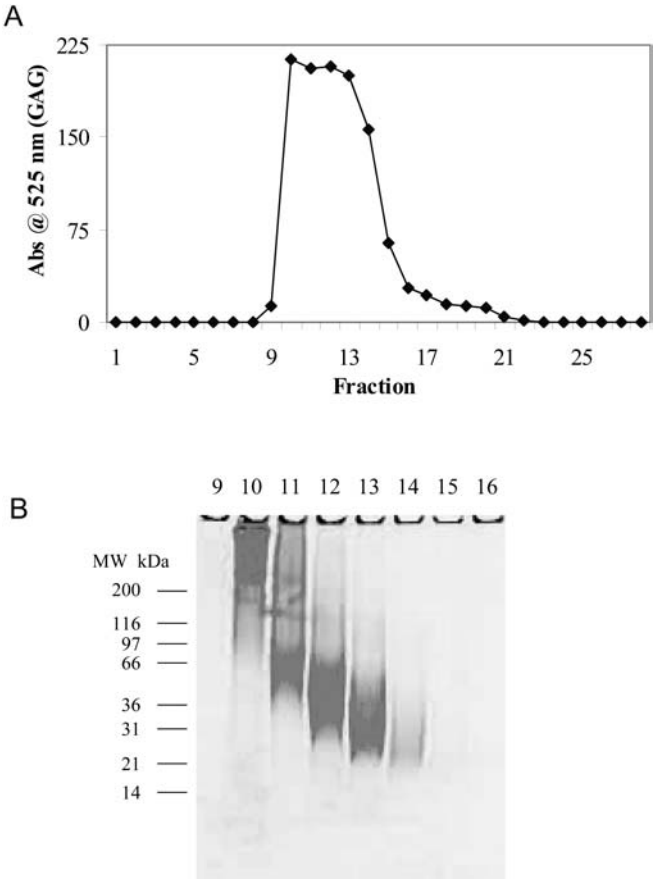

Fig. 4 **A** Size exclusion chromatography on a HiPrep 16/60 Sephacryl S-100 high resolution column of the proteoglycan containing fractions from the HiTrap Q fractionation shown in Fig. 3. The column was eluted with 0.8 M NaCl, 0.1 M sodium acetate, pH 8.0, at a rate of 0.5 ml/min; 4-ml fractions were collected. The GAG content in each fraction was measured by DMMB. **B** SDS/PAGE analysis of the fractions from **A** on 4–20% linear gradient gels stained with Alcian blue. Fractions 10 and 11 contain primarily the large MW PGs while 12–15 contain predominantly small MW PGs

similarity to mammalian tendon. The muscle tendon complex is U-shaped and hence, during contraction, exerts a compressive and shear load on the tendon. As in mammalian tendon this loading regimen is associated with a fibrocartilaginous region that acts as a cushion, lying between the tendon and the jaw element. Tensile forces generated by the muscle will be dissipated by the broad insertion of the tendon. The tissue pull-out of the prismatic cartilage of the jaw is minimized by the oblique angle of the insertion, the multiple layers of calcification and the trabeculae (Summers 2000).

Histological and biochemical analyses demonstrated that the fibrocartilage bears similarities to that found in mammals. Although it is not clear that these tissues are homologous, they are certainly functional analogs. Comparative studies of the biochemistry of shark and mammalian connective tissues are in the early stages (Sakaguchi et al. 2001), but there are significant differences between these two lineages. Our results indicate that the overall GAG contents of the pad and tensile regions of the stingray tendon are comparable to those from the fibrocartilaginous regions of mammalian tendons (Table 1). However, the ratio of total GAG in the compressive region compared to GAG in the tensile regions was one-fifth to one-third of that reported for mammals (Gillard et al. 1977; Okuda et al. 1987; Vogel and Koob 1989).

Based on the results from the glycosidase digestions, the large PG from the stingray tendon contains both chondroitin sulfate and keratan sulfate. This observation suggests that it is an aggrecan-like molecule typically found in the fibrocartilaginous regions of mammalian tendon and predominating in mammalian articular cartilage (Vogel et al. 1994). The relatively high amounts of this PG in the fibrocartilaginous tendon pad would provide compressive stiffness otherwise absent from linear tendon. Moreover, the localization of the large PG-rich fibrocartilaginous pad in regions of the tendon experiencing compression and shear loads in addition to tensile loads implies that the resident tendon cells respond to these loads by producing a mechanically appropriate tissue. If so, the capacity of tendon cells to respond to mechanical loads other than tension arose early in the vertebrate lineage. Decorin is the dermatan sulfate rich small proteoglycan abundant in mammalian tendon that we expected to find in the stingray tendon and fibrocartilage. However, the size of the predominant small proteoglycan from the stingray tendon (20–80 kDa) is significantly less than that of mammalian decorin (100–200 kDa). In addition, while it does contain dermatan sulfate, its GAG chain composition differs from that of decorin in that the relative proportion of chondroitin to dermatan sulfate is substantially greater. The core protein of mammalian decorin is ~35 kDa (Krusius and Ruoslahti 1986). The core protein of the small PG from the stingray tendon appears to be less than 20 kDa. Whether this PG is decorin, a molecular variant of decorin, or a novel proteoglycan specific to elasmobranch tendon can only be determined with further analysis of the core protein.

The gross anatomy and histology of the VIM tendon and associated fibrocartilaginous pad are similar to analogous tendons from mammals and to those documented in anurans (Carvalho and Felisbino 1999). In addition, the large proteoglycan appears quite similar to mammalian aggrecan. In contrast, the smaller, decorin-rich proteoglycans from the ray tendon differ from those in mammals in both size and GAG composition. This could indicate that the component PGs are completely different gene products than those found in mammals, though given the high degree of conservation in connective tissue macromolecules, this seems unlikely. These results reinforce the lack of even the most basic comparative data from non-model system organisms. The comparative biochemistry of shark connective tissues is bound to inform our understanding of other vertebrates and of the evolution of the vertebrate musculoskeletal system.

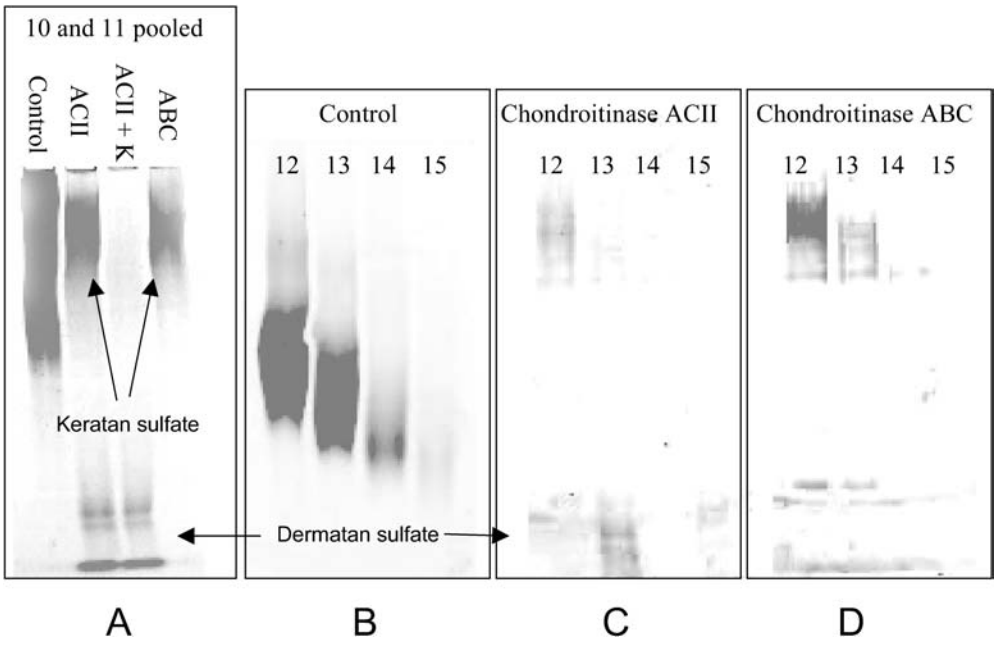

Fig. 5 **A** Glycosidase digests of the pooled extracts from fractions 10 and 11 of the size exclusion chromatography (Fig. 4). Chondroitinase ACII and ABC leave keratan sulfate polymers intact on the core protein, but this band disappears following digestion with keratinases. The dermatan sulfate polymers are unaffected by ACII and keratinase digestion but disappear when treated with chondroitinase ABC. **B** Glycosidase digests of the remaining PG rich fractions. The GAG chains of the small PGs seen in the control lanes are reduced to small fragments of dermatan sulfate by ACII digestion **(C)**, and completely digested by ABC **(D)**. That there are some larger, keratan sulfate containing PGs in fractions 12 and 13 is evidenced by the high MW broad band that survives both ACII and ABC

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