Extensive Heterogeneity of Proteoglycans Bearing Fucose-Branched Chondroitin Sulfate Extracted from the Connective Tissue of Sea Cucumber†

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ABSTRACT: The major sulfated polysaccharide in the sea cucumber body wall is a fucose-branched chondroitin sulfate. This glycosaminoglycan has side-chain disaccharide units of sulfated fucopyranosyl or sulfate esters linked to the O-3 position of the β-D-glucuronic acid residues. These unusual fucose branches and sulfate esters block the access of chondroitinases to the chondroitin sulfate core [Vieira & Mourão (1988) *J. Biol. Chem. 263*, 18176–18183; Vieira et al. (1991) *J. Biol. Chem. 266*, 13530–13536]. We now report the isolation and preliminary characterization of the proteoglycans bearing this unique fucose-branched chondroitin sulfate. They were extracted using guanidine hydrochloride solutions containing protease inhibitors and were purified by anion-exchange and gel-filtration columns. Interestingly, the sea cucumber proteoglycans were cleaved by chondroitinase AC or ABC, indicating that the β-D-glucuronic acid residues close to the reducing end of the polysaccharide chain are neither fucosylated nor sulfated. SDS–polyacrylamide gel electrophoresis revealed several fractions of proteoglycans of different molecular sizes but containing a similar hexuronic acid/protein ratio and a similar type of glycan chain. Possibly, the low-molecular-size fractions arise from a protease cleavage of a larger molecule. In contrast with the results observed for most vertebrate proteoglycans, which contain a single core protein for each type of proteoglycan, chondroitinase AC or ABC releases from the sea cucumber proteoglycans a wide variety of core proteins. These observations are the first detailed study of a proteoglycan from invertebrate tissue and reveal extensive heterogeneity when compared with proteoglycans from vertebrate connective tissue.

During the last few years, we have studied sulfated polysaccharides in different invertebrate connective tissues. The main purpose of such studies is to compare these polysaccharides with the well-known glycosaminoglycans that occur in vertebrate tissues and to relate their structure with physicochemical and biological properties. In previous studies, we have reported the isolation of novel sulfated polysaccharides from the tunic of ascidians (Albano & Mourão, 1983, 1986; Mourão & Perlin, 1987; Pavão et al., 1989a,b, 1990; Albano et al., 1990) and the body wall of the sea cucumber (Mourão & Bastos, 1987; Vieira & Mourão, 1988; Vieira et al., 1991).

The sulfated polysaccharides in the body wall of the sea cucumber occur as three fractions that differ markedly in molecular weight and chemical composition. One fraction contains primarily a sulfated fucan, whereas another fraction, containing a high-molecular-weight component, has a higher proportion of fucose and small amounts of galactose and amino sugars (Mourão & Bastos, 1987; Vieira & Mourão, 1988). The third and largest fraction contains a unique fucose-branched chondroitin sulfate (Vieira & Mourão, 1988; Vieira et al., 1991). This glycosaminoglycan has side-chain disaccharide units of sulfated fucopyranosyl linked to approximately half of the β-D-glucuronic acid moieties through the O-3 position of the acid (Vieira & Mourão, 1988). In addition, the sea cucumber chondroitin sulfate contains sulfate at position O-3 of the glucuronic acid (Vieira et al., 1991). These unusual fucose branches and sulfate esters block the access of chondroitinases to the chondroitin sulfate core. However, after defucosylation and/or desulfation, the sea cucumber chondroitin sulfate is degraded by chondroitinases (Vieira & Mourão, 1988; Vieira et al., 1991).

Glycosaminoglycans occur in vertebrate tissues covalently linked to protein, as proteoglycan molecules (Hascall & Hascall, 1981; Ruoslahti, 1988; Heinegard & Oldberg, 1989). In cartilages, there are at least two distinct molecular species of proteoglycans. One is aggrecan, a large, aggregating chondroitin sulfate/keratan sulfate proteoglycan (Hascall & Hascall, 1981; Ruoslahti, 1988). The other class of cartilaginous proteoglycans includes low-molecular-weight proteoglycans, containing chondroitin sulfate or dermatan sulfate chains (Ruoslahti, 1988; Heinegard & Oldberg, 1989). In addition, several studies suggest that distinct molecular species of protein are involved in the core regions of these proteoglycans and that possibly each tissue or organ has a characteristic composition of chondroitin sulfate proteoglycan subtypes (Kimata et al., 1986; Oohira et al., 1988).

In the present work, we report the isolation of proteoglycans bearing fucose-branched chondroitin sulfate from the connective tissue of sea cucumber. This is the first detailed study of a proteoglycan from invertebrate tissue. Surprisingly, these molecules show a high degree of heterogeneity, differing mainly in the size of the core protein. Possibly, the low-molecular-size species arise from a protease cleavage of a larger proteoglycan in the sea cucumber tissue.

MATERIALS AND METHODS

Materials. The sea cucumber *Ludwigothurea grisea* (Echinodermata–Holothuroidea) was collected in Guanabara Bay (Urca), Rio de Janeiro, and maintained in the laboratory in an aerated aquarium at room temperature. Chondroitinase AC (EC 4.2.2.5) from *Arthrobacter aurescens* and chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris* were from

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Seikagaku Kogyo Company, Ltd (Tokyo, Japan), papain (P-4762) and guanidine hydrochloride were from Sigma Chemical Co. (St. Louis, MO), 1,9-dimethylmethylene blue was from Aldrich Chemical Co. (Milwaukee, WI), DEAE-cellulose (DE-52) was from Whatman Chemical Separation Ltd (England), and dextrans with different molecular weights were from Pharmacia LKB Biotechnology (Uppsala, Sweden). High-molecular-weight proteoglycan from bovine nasal septa (aggrecan) and low-molecular-weight proteoglycan from human articular cartilage (biglycan) were a gift from Dr. N. DiFerrante (Department of Biochemistry, Baylor College of Medicine, Houston, TX) and from Dr. L. O. Sampaio (Department of Biochemistry, Escola Paulista de Medicina, São Paulo, SP, Brazil), respectively.

Extraction of the Proteoglycans from the Connective Tissue of the Sea Cucumber. The body wall of the sea cucumber was quickly separated from the viscera and chilled in dry ice. Thereafter, the outer soft epithelium layer was removed by scraping with a scalpel and the inner muscular layer was manually detached. The clean connective tissue (~20 g, wet weight) was cut into small pieces and immediately immersed in 100 mL of 50 mM sodium acetate buffer (pH 6.0) containing 3 M guanidine hydrochloride, 50 mM EDTA, and the following protease inhibitors (Hascall & Kimura, 1982): 10 mM 6-aminohexanoic acid, 1 mM benzamidine hydrochloride, and 1 mM phenylmethanesulfonyl fluoride. In some experiments, 1% (v/v) Triton X-100, 1.25% (v/v) β-mercaptoethanol, or different concentrations of guanidine hydrochloride were added to the extraction buffer, or 8 M urea replaced guanidine hydrochloride. Proteoglycans were extracted by stirring these mixtures for 24 h at 4 °C. Extracts were clarified by centrifugation (2000*g* for 10 min at 4 °C). The supernatants were stored at −20 °C, and the residues were reextracted with 100 mL of the same solution described above for another 24-h period. The combined guanidine hydrochloride extracts were dialyzed extensively at 4 °C against 6 L of 50 mM sodium acetate buffer (pH 6.0) containing 8 M urea, 50 mM EDTA, and the protease inhibitors described above. This dialyzed guanidine hydrochloride extract was subsequently treated with DEAE-cellulose, as described in the next paragraph. The residues were washed twice with 10 volumes of distilled water to remove guanidine hydrochloride and protease inhibitors, digested with papain, precipitated (Vieira & Mourão, 1988; Vieira et al., 1991), and redissolved in water for determination of hexuronic acid.

Purification of the Proteoglycans Containing Fucose-Branched Chondroitin Sulfate. (*a*) *DEAE-Cellulose.* The dialyzed guanidine hydrochloride extract (100 mL) was mixed with 50 mL of preswollen DEAE-cellulose, equilibrated in the same solution. After 5 min of gentle agitation, the suspension was packed into a glass column (30 × 1.5 cm). Thereafter, the column was washed with 100 mL of the same solution used for dialysis of the proteoglycans and subjected to a linear gradient of 0 → 1 M NaCl in the same solution, as previously described (Vieira & Mourão, 1988). The flow rate of the column was 12 mL/h, and fractions of 3 mL were collected. They were assayed by the carbazole reaction (Dische, 1947) for hexuronic acid, by the Dubois reaction (Dubois et al., 1956) for total hexoses, by metachromatic assay using 1,9-dimethylmethylene blue (Farndale et al., 1986), and by absorbance at 280 nm. The NaCl concentration was estimated by conductivity. The fractions containing the proteoglycans of the fucose-branched chondroitin sulfate, as indicated by a positive carbazole test, were pooled and dialyzed against the same solution described above. This sample was applied to a newly packed DEAE-cellulose column (30 × 1.5 cm) and repurified as described above, except that an NaCl gradient of 0 → 0.75 M was used. The fractions of this second column containing the proteoglycans of the fucose-branched chondroitin sulfate were pooled, dialyzed against distilled water, and lyophilized (~20 mg).

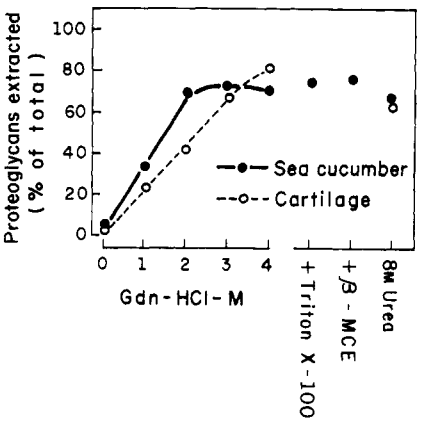

FIGURE 1: Comparison of extraction of the proteoglycans from sea cucumber and from cartilage using chaotropic solvents. The catch connective tissue from sea cucumber (●) or bovine articular cartilage (○) (2 g wet weight) was mixed with 20 mL of 50 mM sodium acetate buffer (pH 6.0), containing 50 mM EDTA, protease inhibitors (see Materials and Methods) and increasing concentrations of guanidine hydrochloride (Gnd-HCl). In some experiments (indicated on the abscissa), 1% (v/v) Triton X-100 or 1.25% (v/v) β-mercaptoethanol (β-MCE) was added to the extraction buffer containing 4 M guanidine hydrochloride, or 8 M urea replaced guanidine hydrochloride. Proteoglycans were extracted by stirring for 24 h at 4 °C. Extracts were clarified by centrifugation and dialyzed against distilled water (*extracted proteoglycans*). The residues, containing *nonextracted proteoglycans*, were washed twice with 10 volumes of distilled water to remove protease inhibitors and the extraction buffer. Sulfated polysaccharides were extracted from the proteoglycan solutions or from the residual tissues by papain digestion, purified by cetyl pyridinium chloride precipitation (Vieira et al., 1991) and dissolved in distilled water, and the amounts of extracted and nonextracted proteoglycans were estimated in these solutions by the carbazole reaction.

(*b*) *Sephacryl S-400.* Ten milligrams of the lyophilized proteoglycans from the second NaCl gradient were dissolved in 1 mL of 50 mM sodium acetate buffer (pH 6.0), containing 8 M urea, 0.5 M NaCl, and the protease inhibitors. Thereafter, the mixture was applied to a Sephacryl S-400 column (100 × 0.9 cm), previously calibrated with dextrans of different average molecular weights. The column was equilibrated and eluted with the same solution used to dissolve the proteoglycans, at a flow rate of 4 mL/h. Fractions of 1 mL were collected and assayed by the carbazole reaction, by the metachromatic assay, and by absorbance at 280 nm. The fractions containing the proteoglycans of the fucose-branched chondroitin sulfate, as indicated by a positive carbazole test, were pooled, dialyzed against distilled water, and lyophilized, yielding 5.5 mg of purified proteoglycans.

All purification procedures, including columns and dialysis, were performed at 4 °C to prevent degradation of the proteoglycans.

Enzymatic Digestion. (*a*) *Incubation with Chondroitinase AC or ABC.* Proteoglycans (100 μg) were incubated with 10 milliunits of chondroitinase AC or chondroitinase ABC (Saito et al., 1968) in 50 mM ethylenediamine/acetate buffer (pH 8.0) at 37 °C for 2 h. In some experiments, the following protease inhibitors were added to the incubation mixtures: 10 mM 6-aminohexanoic acid, 1 mM benzamidine hydrochloride, and 1 mM phenylmethanesulfonyl fluoride.

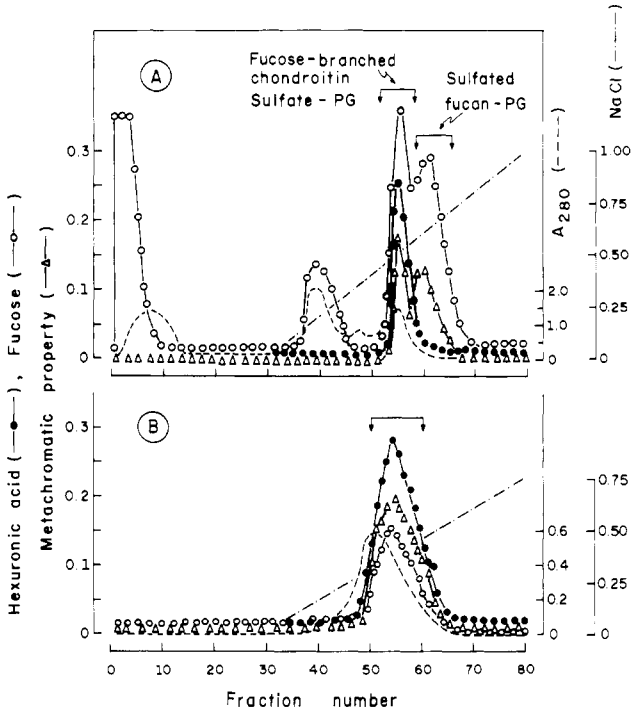

FIGURE 2: Anion-exchange chromatography of the macromolecular material extracted by 3 M guanidine hydrochloride from the sea cucumber connective tissue. (A) The macromolecular material was chromatographed on a DEAE-cellulose column, as described in Materials and Methods. The fractions were eluted with a linear gradient of NaCl, from 0 to 1 M in 50 mM sodium acetate buffer (pH 6.0) containing 8 M urea, 50 mM EDTA, and a mixture of protease inhibitors. They were assayed by the carbazole reaction (●), by the Dubois reaction (○), by metachromatic assay (△) and by absorbance at 280 nm (---). The NaCl concentration was estimated by conductivity (– • –). The fractions indicated by brackets, containing sulfated proteofucans, were pooled and analyzed further in Figure 4. (B) The proteoglycans bearing fucose-branched chondroitin sulfate (left bracket) were pooled and dialyzed against the same buffer solution containing urea, described above, applied to a new packed DEAE-column (30 × 1.5 cm), and repurified as described above, except that the NaCl gradient was from 0 to 0.75 M. Values of the carbazole and Dubois reactions are indicated on the ordinate as absorbance and therefore do not indicate the fucose/hexuronic acid molar ratios. Molar ratios are given in Table I. Smaller volumes were used for the Dubois reaction in (B) than in (A), which explains the lower values for fucose absorbance in (B).

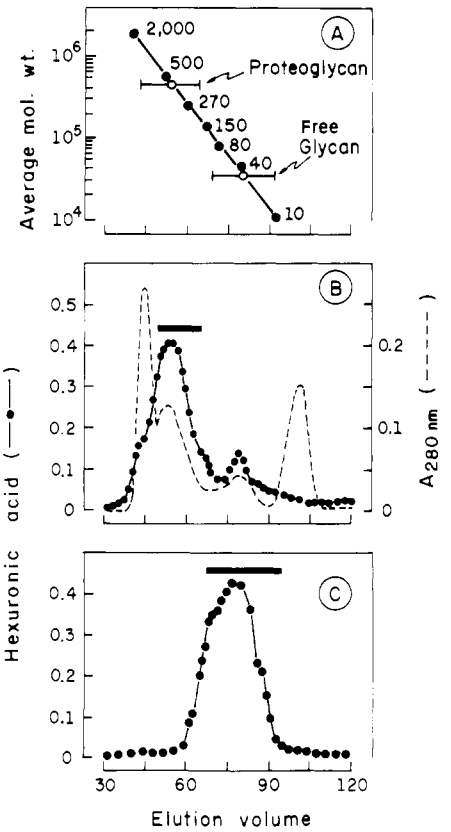

FIGURE 3: Sephacryl S-400 chromatography of standard dextrans (A) and of the sea cucumber proteoglycans before (B) and after β-elimination (C). The proteoglycans purified by DEAE-cellulose or standard dextrans (10 mg) were applied to a 100 × 0.9 cm column of Sephacryl S-400 eluted at 4 °C at a flow rate of 4 mL/h with 50 mM sodium acetate buffer (pH 6.0) containing 8 M urea, 50 mM EDTA, 0.5 M NaCl, and protease inhibitors (see Materials and Methods). Fractions of 1 mL were collected and assayed by the carbazole reaction (●) and by absorbance at 280 nm (---). The fractions containing the proteoglycans and the free glycan, as indicated by brackets in (B) and (C), respectively, were pooled, dialyzed against distilled water, and lyophilized. The elution of standard dextrans was followed by the Dubois reaction, and the values in (A) indicate their average molecular masses as kilodaltons. Open circles and horizontal bars in (A) indicate the average and range molecular weight of the proteoglycan from sea cucumber and of the glycan releases by β-elimination.

(*b*) *Incubation with Papain.* Proteoglycans (100 μg) were incubated for 2 h at 60 °C with 1 μg of papain in 100 μL of 100 mM sodium acetate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine.

(*c*) *Incubation with Trypsin.* Proteoglycans (100 μg) were incubated at 25 °C with 5 μg of trypsin in 100 μL of phosphate buffer (pH 7.4) containing 0.15 M NaCl and 50 mM EDTA.

At the completion of all reaction periods, 5 μL of 30 mM Tris-HCl buffer (pH 6.6) containing 5% (v/v) glycerol, 0.02% SDS, and 0.01% bromophenol blue were added and the mixtures were heated in boiling water for 1 min.

β-Elimination. Proteoglycans (1 mg) were dissolved in 1 mL of 100 mM NaOH containing 100 mM $NaBH_4$ and incubated at 25 °C for 10 h. The reaction mixture was dialyzed against distilled water, lyophilized, and dissolved in the same solution used to stop the enzymatic digestions.

Electrophoretic Procedures. (*a*) *SDS–Polyacrylamide Gel Electrophoresis.* Intact and cleaved proteoglycans (~50 μg) were separated on 6 × 8 × 0.075 cm gels with a 2.5–20% linear gradient of acrylamide in 0.1% SDS, using a 2.5% stacking gel (Laemmli, 1970). The gels were run on a Bio-Rad vertical gel electrophoresis apparatus at 30 mA per gel (~100 V). After electrophoresis, the gels were stained either with silver (Merril, 1984) or with 0.1% toluidine blue in 1% acetic acid (Vieira & Mourão, 1988). The molecular mass standards (Sigma) were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

(*b*) *Agarose Gel Electrophoresis.* This procedure was used for identification of the various sulfated polysaccharides extracted from the sea cucumber, as previously described (Vieira & Mourão, 1988). Polysaccharides (25 μg) were applied to a 0.5% agarose gel in 50 mM 1,3-diaminopropane/acetate buffer (pH 9.0) and run at 110 V for 1 h. The sulfated polysaccharides in the gels were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for 12 h and then dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v).

Chemical Methods. Hexuronic acid was measured by the carbazole reaction (Dische, 1947), methylpentose by the method of Dische and Shettles (1948), total hexose by the Dubois et al. reaction (1956), and protein by the method of Lowry et al. (1951).

Table I: Purification of the Proteoglycans Containing Fucose-Branched Chondroitin Sulfate

step	total protein (mg)	total hexuronic acid (mg)	total fucose (mg)	hexuronic acid/protein ratio (mg/mg)	fucose/hexuronic acid ratio (mol/mol)	total recovery[a] (%)	purification[b] (fold)
Gdn-HCl extract	91.2	2.82	23.4	0.030	9.81	100	1.0
first DEAE-cellulose	5.8	1.08	5.0	0.186	5.48	38	6.2
second DEAE-cellulose	3.1	0.68	1.5	0.219	2.61	24	7.3
Sephacryl S-400	1.4	0.34	0.6	0.242	2.08 (1.77)[c]	12	8.1

[a] Calculation of the proteoglycan recovery is based on the total hexuronic acid. [b] Purification index is based on the hexuronic acid/protein ratio. [c] Molar ratio of fucose/hexuronic acid observed for the fucose-branched chondroitin sulfate (Mourão & Bastos, 1987).

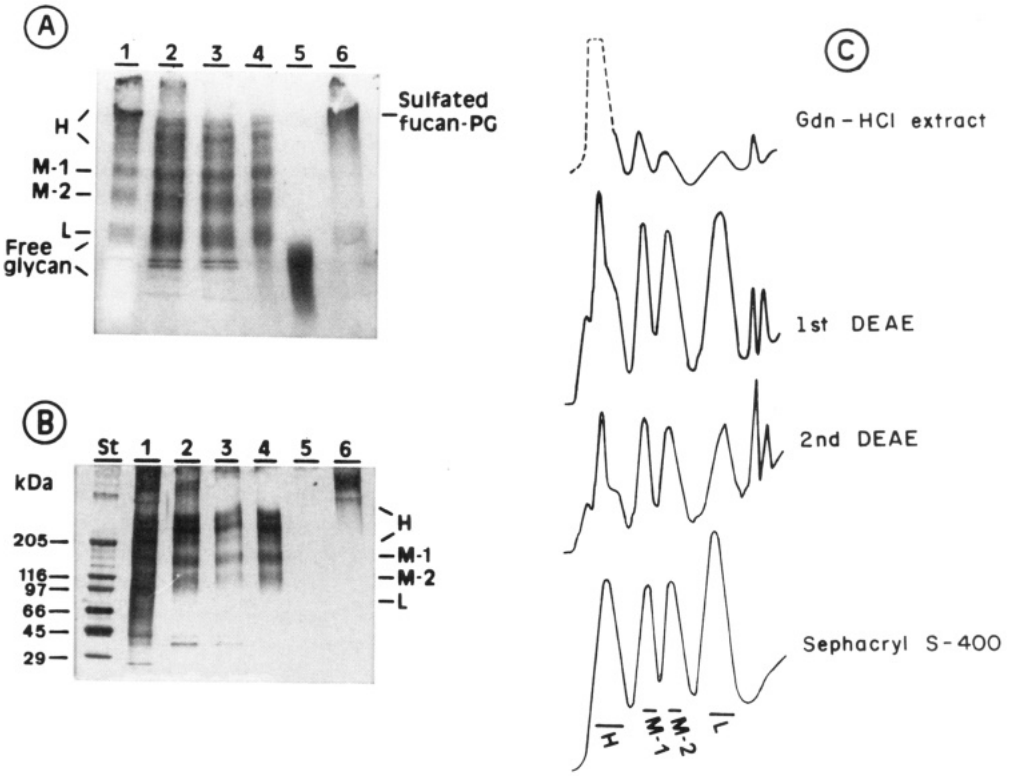

FIGURE 4: SDS–polyacrylamide gel electrophoresis of the proteoglycans extracted from the sea cucumber connective tissue at various stages of purification. Samples (~50 μg dry weight) were applied to SDS–polyacrylamide gels with a 2.5–20% linear gradient of acrylamide and run on a vertical electrophoresis apparatus at 30 mA per gel. After electrophoresis, the gels were stained with toluidine blue (A) or silver (B). The samples are 3 M guanidine hydrochloride extract from sea cucumber (lane 1), proteoglycans purified in the first (lane 2) and second (lane 3) DEAE-column (see Figure 2), and on Sephacryl S-400 before (lane 4) and after (lane 5) β-elimination (see Figure 3B,C), and the sulfated proteofucan (lane 6) partially purified in the DEAE-column (Figure 2A). (C) shows densitometry of lanes 1, 2, 3, and 4 stained with toluidine blue. Dashed line in the tracing labeled Gdn-HCl extract indicates the electrophoretic peak of the sulfated proteofucan, which contaminates the high-molecular-weight fraction of the proteoglycans bearing fucose-branched chondroitin sulfate. The dashed line was deduced from comparison between the electrophoretic mobility of the sulfated proteoglycans in the guanidine hydrochloride extract and that of the purified sulfated proteofucan (lane 6, panel A). In (A) and (C), H, M-1, M-2, and L indicate the electrophoretic migration of the sea cucumber proteoglycans with high, medium-1, medium-2, and low molecular weight, respectively.

RESULTS

Extraction of the Proteoglycans Bearing Fucose-Branched Chondroitin Sulfate with Chaotropic Solvents. Proteoglycans were extracted from the connective tissue of sea cucumber with increasing concentrations of guanidine hydrochloride (Figure 1, closed circles). Since the fucose-branched chondroitin sulfate is the only hexuronic acid-containing polysaccharide found in the sea cucumber body wall (Vieira & Mourão, 1988), the extraction of the proteoglycans was followed by the carbazole reaction. The extraction of proteoglycans bearing fucose-branched chondroitin sulfate increased sharply with the concentration of guanidine hydrochloride up to 2 M and thereafter remained constant. Addition of 1% Triton X-100 or 1.25% β-mercaptoethanol to the guanidine hydrochloride solution or replacement of guanidine hydrochloride by 8 M urea did not increase the amount of extracted proteoglycans. In a control experiment with bovine articular cartilage (Figure 1, open circles), the fraction of total proteoglycans in the tissue that was extracted by guanidine hydrochloride increased up to 4 M. Nevertheless, the proportions of total proteoglycans extracted with high concentrations of guanidine hydrochloride were approximately the same in the two tissues.

Purification of the Proteoglycans Bearing Fucose-Branched Chondroitin Sulfate. The material extracted from the sea cucumber connective tissue by 3 M guanidine hydrochloride was separated by DEAE-cellulose into a single hexuronic acid-containing peak at ~0.5 M NaCl (Figure 2A). A second peak, eluted at higher concentrations of NaCl, was positive for the Dubois and metachromatic reactions but not for the hexuronic acid test and corresponds to the sulfated proteofucans, as identified by agarose gel electrophoresis (not shown). In addition, there were large amounts of proteins and materials sensitive to the Dubois reaction that were not bound to the column or were eluted at low NaCl concentrations. The hexuronic acid-containing fractions were pooled and repurified

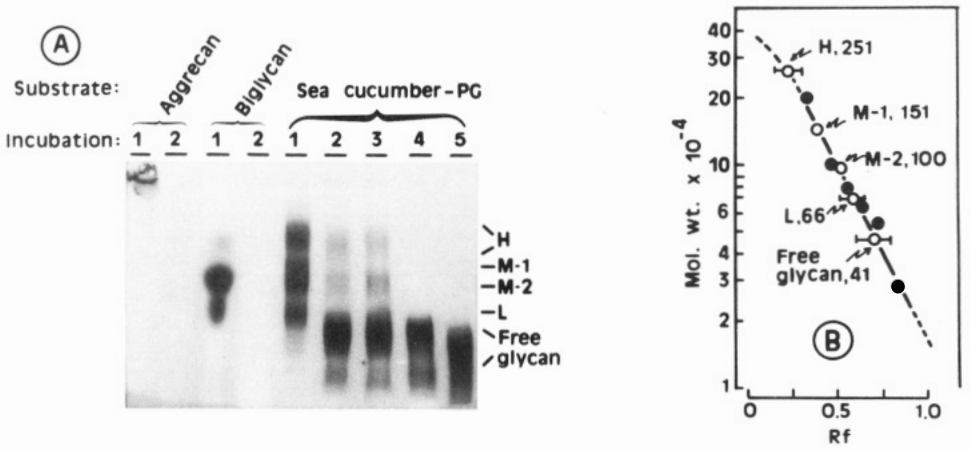

FIGURE 5: (A) SDS–polyacrylamide gel electrophoresis of aggrecan, biglycan, and sea cucumber proteoglycans before (1) and after incubation with chondroitinase ABC (2), chondroitinase AC (3), papain (4), or β-elimination (5). Proteoglycans (~50 μg) were applied to an SDS–polyacrylamide gel and electrophoresed as described for Figure 4. After electrophoresis, the proteoglycans were stained with toluidine blue. (B) shows estimation of the molecular weight of sea cucumber proteoglycans with high (H), medium-1 (M-1), medium-2 (M-2), and low (L) molecular weight and of the chondroitinase ABC-released glycan by SDS–polyacrylamide gel electrophoresis. The standard proteins (closed circles) are carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa). The average molecular masses of the sea cucumber proteoglycans and of the free glycan (open circles) are indicated in the figure as kilodaltons.

in another DEAE-cellulose column, yielding a homogeneous peak (Figure 2B).

The hexuronic acid-containing fraction was purified further by gel filtration on Sephacryl S-400 (Figure 3B), previously calibrated with dextrans of different average molecular weights (Figure 3A). The proteoglycans eluted as a broad, symmetrical peak, with an average molecular mass of ~500 kDa (compared with dextrans), separated from two peaks of UV-absorbing material that eluted at the V_0 and near the V_t of the column. A small amount of free glycan (≤5% of the total) was also identified in the gel chromatography (Figure 3A,B).

To confirm that the main peak containing hexuronic acid is a proteoglycan bearing fucose-branched chondroitin sulfate, the purified fraction was chromatographed on Sephacryl S-400 after β-elimination (Figure 3C). The main hexuronic acid peak was shifted by this treatment, so that it eluted with an average molecular weight of ~40 kDa (compared with dextrans). Analysis of this material by agarose gel electrophoresis revealed exclusively fucose-branched chondroitin sulfate (not shown).

Table I summarizes the yield of proteoglycans bearing fucose-branched chondroitin sulfate, on the basis of the amounts of hexuronic acid, during the purification processes. The hexuronic acid/protein ratio increases markedly while the fucose/hexuronic acid ratio decreases during the various steps of purification, reflecting the separation of proteoglycans from contaminant proteins and from other glycans containing fucose, respectively. In fact, the proteoglycans obtained after gel filtration (Table I) have a fucose/hexuronic acid ratio similar to that reported previously for fucose-branched chondroitin sulfate, when this was extracted using papain and purified by anion exchange and gel filtration.

The purity of the proteoglycans was assessed using SDS–polyacrylamide gel electrophoresis and staining with toluidine blue (Figure 4A) and with silver (Figure 4B). Surprisingly, the proteoglycans bearing fucose-branched chondroitin sulfate show several distinct bands on SDS–polyacrylamide gels. These were grouped roughly into high (H), medium 1 (M-1), medium 2 (M-2), and low (L) molecular weight proteoglycans, as indicated in Figure 4A. The high-molecular-weight fraction occasionally separates into distinct electrophoretic bands on polyacrylamide gels. This fraction comigrates with a sulfated

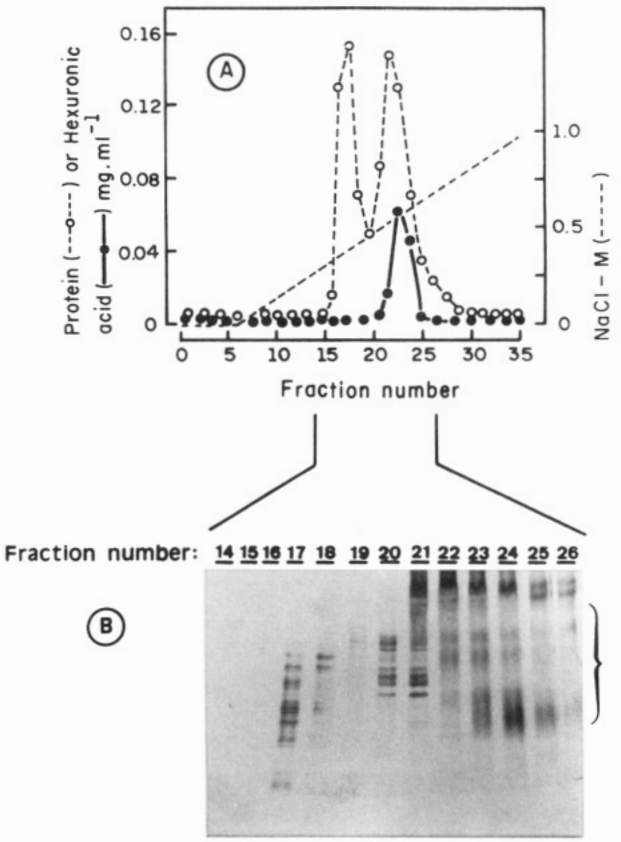

FIGURE 6: Analysis of the products formed by chondroitinase ABC digestion of the sea cucumber proteoglycans. (A) Sea cucumber proteoglycans (5 mg) were incubated with chondroitinase ABC (0.5 unit) in 1 mL of 50 mM ethylenediamine/acetate buffer (pH 8.0). After incubation at 37 °C for 2 h, the mixture was applied to a DEAE-cellulose column (5 × 0.5 cm), washed with 10 mL of a 50 mM sodium acetate buffer (pH 6.0), and subjected to a linear gradient of 0 → 1 M NaCl in the same buffer, as previously described. The flow rate of the column was 10 mL/h, and fractions of 1 mL were collected. They were assayed by the Lowry (○) and carbazole (●) reactions. The NaCl concentration (---) was estimated by conductivity. (B) fractions from (A) were electrophoresed on an SDS–polyacrylamide gel (see legend of Figure 4) and stained sequentially with silver and then with toluidine blue. The bracket at the right in (B) indicates the electrophoretic bands of fractions 22–25 which are stained by toluidine blue.

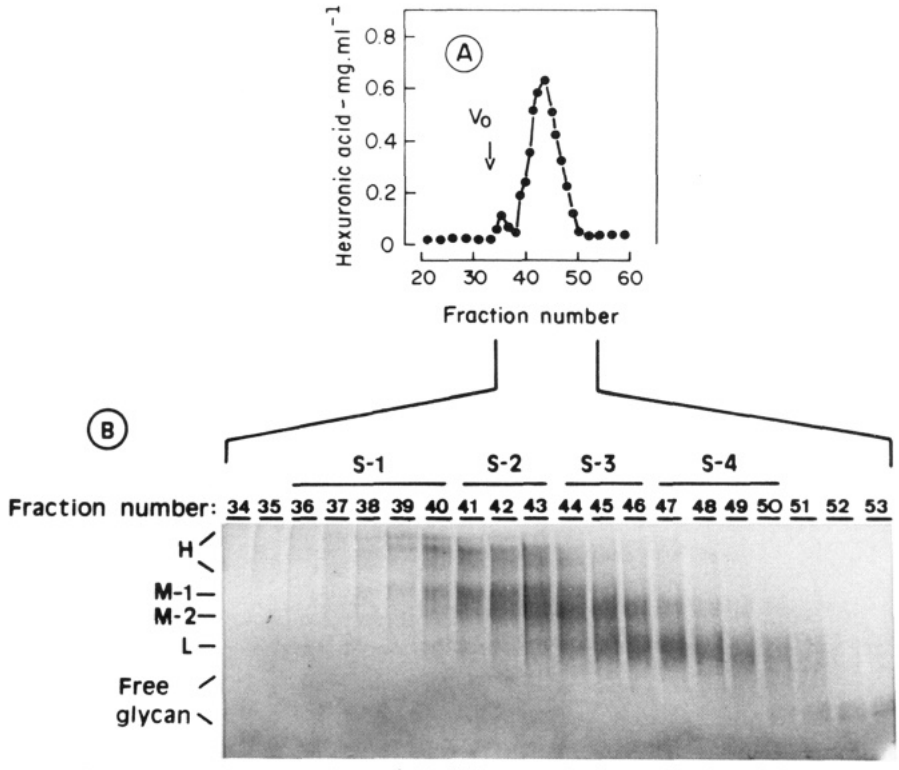

FIGURE 7: Partial separation of the sea cucumber proteoglycans with different molecular weights by gel chromatography on Sephacryl S-400. (A) Gel chromatography of the purified sea cucumber proteoglycans (5 mg) was performed as described in the legend of Figure 3. (B) Fractions from (A) were electrophoresed on an SDS–polyacrylamide gel (see legend of Figure 4) and stained with toluidine blue. H, M-1, M-2, and L indicate the electrophoretic mobilities of proteoglycans with high, medium-1, medium-2, and low molecular weight, respectively. Fractions were pooled as indicated by brackets in (B) into subfractions S-1, S-2, S-3, and S-4 (see text).

proteofucan, which was separated on the first DEAE-column (see lane 6, Figure 4A, and broken line on densitometric scans of polyacrylamide gels shown in Figure 4C).

It is unlikely that the proteoglycans bearing fucose-branched chondroitin sulfate are degraded during the purification procedures, since the relative proportions of the various fractions remain the same throughout (see Figure 4A and densitometric scans of the SDS–polyacrylamide gel electrophoresis shown in Figure 4C). Addition of 1.25% β-mercaptoethanol to the running buffer of the SDS–polyacrylamide gel did not alter the electrophoretic pattern of these proteoglycans, virtually excluding that there are molecules linked by disulfide bonds (not shown).

Staining with silver (Figure 4B) showed a marked decrease in contaminant proteins during the various steps of purification, as expected. Bands coincident with those stained with toluidine blue are observed in the purified proteoglycans. However, the intensities of these bands decrease markedly with molecular weight.

Removal of the Fucose-Branched Chondroitin Sulfate from Proteoglycans by Chondroitinases. Incubation with chondroitinase AC or ABC has been widely used for the removal of chondroitin sulfate chains from proteoglycans and consequently for determining the molecular weight of core proteins (Oohira et al., 1988; Haynesworth et al., 1987; Trotter & Koob, 1989; Oohira et al., 1991). However, the presence of α-L-fucose branches and sulfate esters linked through the O-3 position of the β-D-glucuronic acid residues obstructs the access of chondroitinases to the sea cucumber chondroitin sulfate (Vieira & Mourão, 1988; Vieira et al., 1991). Therefore, we anticipated that this procedure could not be used for the proteoglycans bearing fucose-branched chondroitin sulfate.

Surprisingly, although they were not degraded into disaccharides by chondroitinases, the sea cucumber proteoglycans in fact released from the protein core by chondroitinase AC or ABC degradation a glycan with the same molecular weight as that obtained by β-elimination or papain digestion (Figure 5A). Possibly, disaccharide units at the reducing ends of the sea cucumber chondroitin sulfate, where they are attached to the protein core, are neither fucosylated nor sulfated at O-3 position of the β-D-glucuronic acid and therefore are cleaved by chondroitinases. In contrast to aggrecan and biglycan, whose chondroitin sulfate chains are cleaved by chondroitinase ABC to disaccharides and therefore are no longer detected on SDS–polyacrylamide gels, the glycan released from sea cucumber proteoglycans resists further chondroitinase degradation (Figure 5A). A small amount of the sea cucumber proteoglycans is not cleaved by chondroitinase AC or ABC even after the addition of more enzyme to the incubation mixtures, suggesting the lack of chondroitinase-sensitive disaccharides at the reducing end.

The chondroitinase experiment included a control (not shown), which demonstrates the formation of the same products by chondroitinase ABC digestion of the proteoglycans in the presence or absence of protease inhibitors, to ensure that the enzyme was not contaminated with protease.

On SDS–polyacrylamide gels (Figure 5B), the electrophoretic mobilities of the sea cucumber proteoglycans and of the glycan released by chondroitinases indicate average molecular masses of 251, 151, 100, and 66 kDa for the proteoglycans of high, medium-1, medium-2, and low molecular weight, respectively, and of 41 kDa for the free glycan (compared with globular proteins).

Attempts to identify the core protein of the sea cucumber proteoglycans by SDS–polyacrylamide gel electrophoresis after chondroitinase digestion were unsuccessful due to contamination with the chondroitinase-resistant proteoglycans. In a different attempt we separated the proteins released by

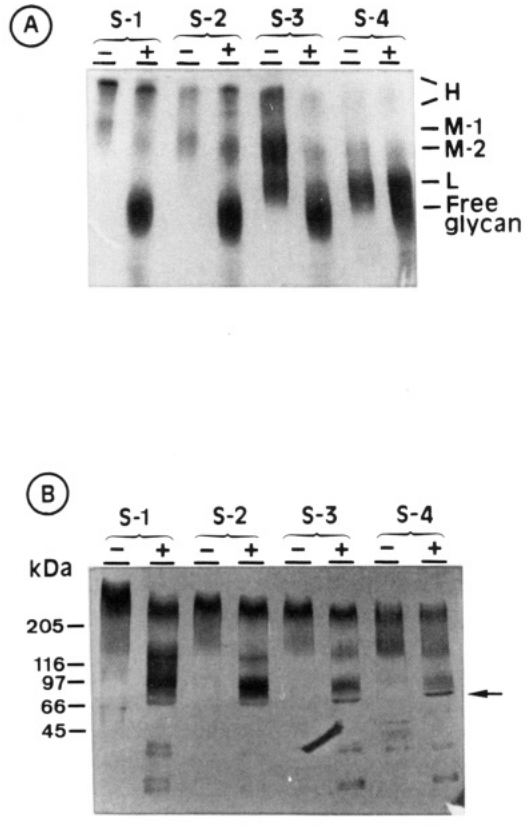

FIGURE 8: SDS–polyacrylamide gel electrophoresis of the products formed by chondroitinase ABC digestion of subfractions S-1, S-2, S-3, and S-4. The subfractions obtained by gel filtration on Sephacryl S-400 (see Figure 7) (~50 μg) were incubated with 50 milliunits of chondroitinase ABC (+) in 50 mM ethylenediamine/acetate buffer (pH 8.0). Appropriate controls were incubated in the absence of enzyme (–). After incubation at 37 °C for 2 h, the mixtures were electrophoresed on an SDS–polyacrylamide gel and stained with toluidine blue (A) or silver (B). The arrow at the right in (B) indicates the electrophoretic mobility of a contaminant protein from chondroitinase ABC.

chondroitinase ABC from free glycan and from chondroitinase-resistant proteoglycans using a DEAE-cellulose column (Figure 6A). Fractions that were positive for protein but not for hexuronic acid (fractions 17–20, Figure 6A), possibly containing core proteins free of glycan chains, exhibited a wide variety of thin protein bands on SDS–polyacrylamide gel electrophoresis (Figure 6B). In contrast, fractions that were positive for hexuronic acid (fractions 22–25, Figure 6A) showed on SDS–polyacrylamide gels several toluidine blue-positive bands, which correspond to the chondroitinase-resistant proteoglycans and to the free glycan (see region indicated by bracket on the right side of fractions 22–25, Figure 6B). Overall, this experiment suggests that proteoglycans from sea cucumber do not have a defined protein core such as that observed for most mammalian proteoglycans (Ruoslahti, 1988; Heinegard & Oldberg, 1989; Kimata et al., 1986; Oohira, 1988) but a wide variety of core proteins with different sizes.[1]

Partial Separation of the Sea Cucumber Proteoglycans According to Molecular Size. A possible explanation for the heterogeneity of the sea cucumber proteoglycans is diversity in the protein core—that is, the several fractions observed on SDS–polyacrylamide gels (Figures 4 and 5) might differ by having protein cores of different sizes. In order to investigate this possibility, the sea cucumber proteoglycans were separated into several subfractions by gel chromatography on Sephacryl S-400 (Figure 7A). Although it was not possible to obtain purified fractions of high, medium-1, medium-2, and low molecular weight, the proteoglycans were subdivided in four different subfractions, denominated S-1, S-2, S-3, and S-4, containing proteoglycans of decreasing molecular size (Figure 7B).

Table II: Hexuronic Acid/Protein Ratios in the Various Subfractions of Sea Cucumber Proteoglycans Obtained by Gel Filtration Chromatography

subfraction[a]	hexuronic acid/protein ratio[b]	subfraction[a]	hexuronic acid/protein ratio[b]
S-1	0.22	S-3	0.27
S-2	0.19	S-4	0.18

[a] See Figure 7. [b] Hexuronic acid and protein were estimated by the carbazole and Lowry reactions, respectively.

Incubation with chondroitinase ABC released from the various subfractions sulfated glycan with the same molecular size (Figure 8A). However, in subfractions S-1 and S-2, there was a preponderance of free core proteins of higher molecular weight than in S-3 and S-4 (Figure 8B). Therefore, it appears that the various fractions of sea cucumber proteoglycans differ in the size of their core proteins. Furthermore, those containing a longer protein core also have more glycan chains attached to them, since the hexuronic acid/protein ratios did not differ significantly among the various subfractions (Table II).

Trypsin Digestion of the Sea Cucumber Proteoglycans. The occurrence of proteoglycans that contain the same type of glycan chain but differ in the size of their core protein suggests that the low-molecular-weight fractions may arise from protease cleavage of a high-molecular-size component. In order to test this possibility, the subfraction S-1 was incubated with trypsin for different times (Figure 9A). Interestingly, trypsin digestion produces proteoglycans similar to the medium-1, medium-2, and low size fractions. In fact, after a 1-min or a 10-min incubation period there is a noticeable decrease of the high-molecular-weight fraction and an increase of the medium-2 fraction (shaded peaks, Figure 9B); after 30 min there is a marked increase of the medium-2 fraction and after 24 h of incubation even the medium-2 component decreases to generate the low-molecular-weight fraction and free glycan. Overall, this experiment reinforces the possibility that the various fractions of sea cucumber proteoglycans arise in vivo from protease cleavage of a large molecule.

DISCUSSION

Figure 10 shows a hypothetical structure for the proteoglycans bearing fucose-branched chondroitin sulfate from sea cucumber connective tissue. The polysaccharide portion of these proteoglycans has a chondroitin sulfate-like structure but contains disaccharide units of sulfated fucopyranosyl or sulfate esters linked to the D-glucuronic acid moieties through the O-3 position of the acid (Vieira & Mourão, 1988; Vieira et al., 1991). These unusual fucose branches and sulfate esters obstruct the access of chondroitinase to the chondroitin sulfate core (*chondroitinase-resistant region*, Figure 10). However, most of the β-D-glucuronic acid residues close to the reducing end of this polysaccharide must be neither fucosylated nor

[1] In a control experiment, we analyzed the core protein released from the small molecular weight proteoglycan of human articular cartilage by chondroitinase ABC. A major protein with 40 kDa was detected by silver staining on SDS–polyacrylamide gels, as expected (Sampaio et al., 1988). Therefore, the wide variety of core proteins released from sea cucumber proteoglycans by chondroitinase ABC cannot be attributed to an artifact of our experimental protocol.

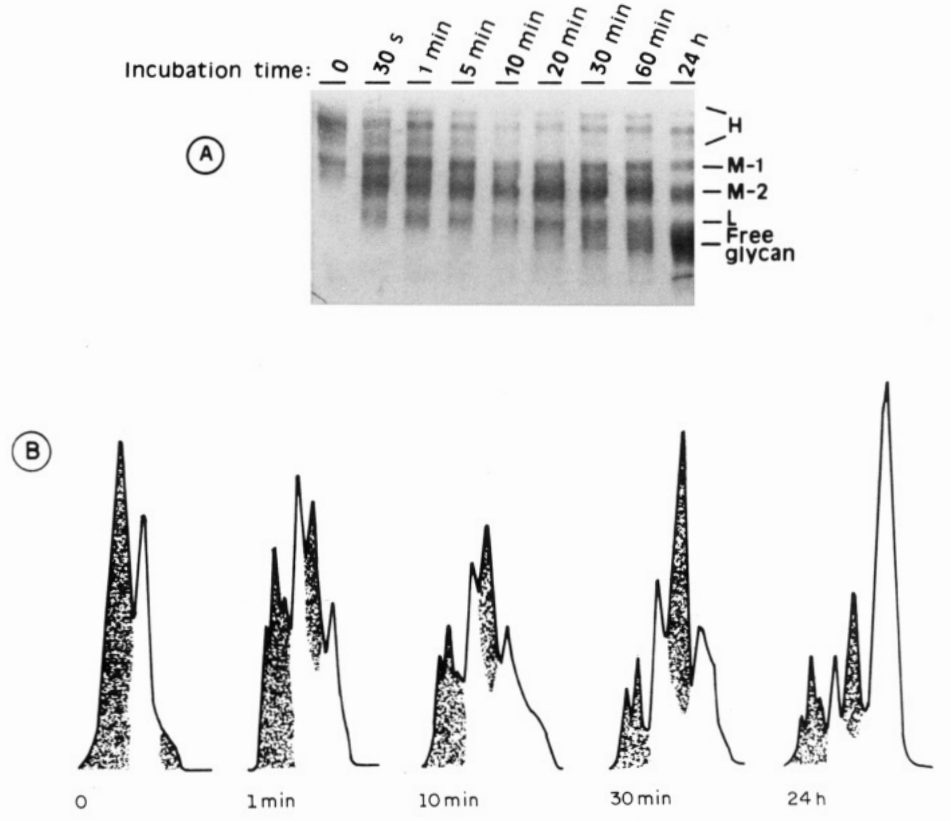

FIGURE 9: Tryptic digestion of the sea cucumber proteoglycans. (A) Subfraction S-1 (100 μg) of the sea cucumber (Figure 7) was incubated with 5 μg of trypsin in 100 μL of phosphate buffer (pH 7.4) containing 0.15 M NaCl and 50 mM EDTA at 25 °C. The reaction was stopped at different times by the addition of 5 μL of 30 mM Tris-HCl buffer (pH 6.6), containing 5% (v/v) glycerol, 0.02% SDS, and 0.01% bromophenol blue to the reaction tube and warming in boiling water for 1 min. (B) shows densitometry of selected lanes from (A) at 0, 1 min, 10 min, 30 min, and 24 h of incubation. The electrophoretic bands which correspond to the high and medium-2 molecular weight fractions of the sea cucumber proteoglycans are shaded in (B).

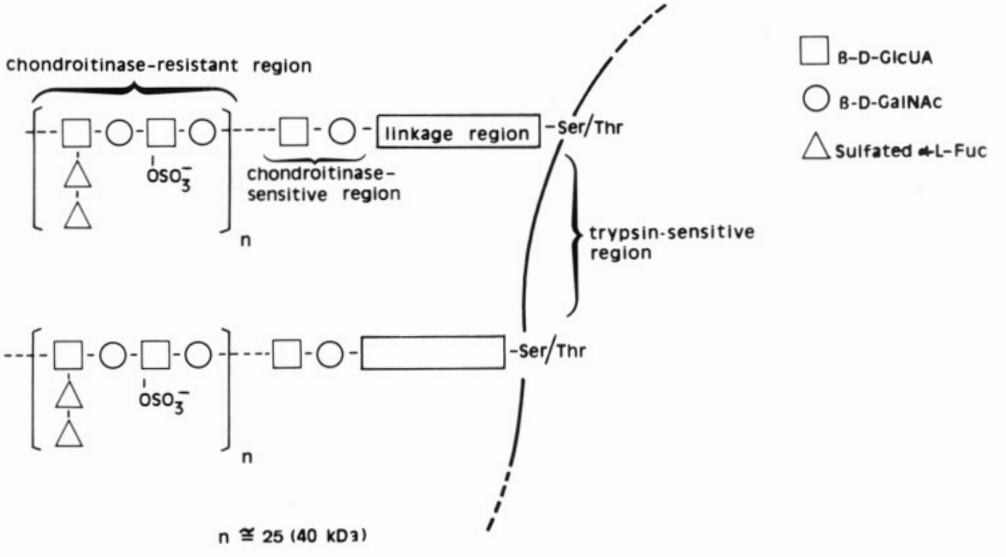

FIGURE 10: Hypothetical structure for the proteoglycans bearing fucose-branched chondroitin sulfate from the catch connective tissue of sea cucumber. Proteoglycans from sea cucumber have chains of chondroitin sulfate-like polysaccharide but contain disaccharide units of sulfated fucopyranosyl units or sulfate esters linked to β-D-glucuronic acid. These unusual fucose branches and sulfate esters obstruct the access of chondroitinase to the chondroitin sulfate core (*chondroitinase-resistant region*). However, most of the β-D-glucuronic acid residues close to the reducing end of this polysaccharide are neither fucosylated nor sulfated (*chondroitinase-sensitive region*). We have no data on the structure of the *linkage region* of the polysaccharide to the core protein, and for the proposition shown in the figure we assume that it is similar to that of chondroitin sulfate proteoglycans from vertebrates. These proteoglycans are cleaved by proteases (*trypsin-sensitive region*) yielding the wide variety in molecular size of their core proteins. See text for other details.

sulfated (*chondroitinase-sensitive region*, Figure 10), since chondroitinase AC or ABC degrades most of the sea cucumber proteoglycans, producing a glycan with approximately the same molecular weight as that released by β-elimination (Figure 5). The glycan is released from the sea cucumber proteoglycans by β-elimination (Figures 3C and 5A), and small amounts of galactose were detected in the fucose-branched chondroitin sulfate extracted from sea cucumber by protease (Vieira & Mourão, 1988). Therefore, we suppose that the polysaccharide is O-linked to the peptide core and

that galactose may be a component of the linkage region. However, we have no data on the structure of the linkage region of these proteoglycans, and for the proposition shown in Figure 10, we assume that it is similar to that of chondroitin sulfate proteoglycans from vertebrates (Hascall & Hascall, 1981; Ruoslahti, 1988; Heinegard & Oldberg, 1989).

Proteoglycans bearing fucose-branched chondroitin sulfate occur in the sea cucumber connective tissue as fractions of different size (Figure 4), which do not differ significantly in their hexuronic acid/protein ratio (Table II). Therefore, the heterogeneity of the sea cucumber proteoglycans cannot be ascribed to a single core protein containing different numbers of glycan chains, as reported for some small proteoglycans from cartilage (Sampaio et al., 1988). In addition, it was not possible to isolate from the sea cucumber proteoglycans a single core protein, as reported for some chondroitin sulfate proteoglycans from cartilage (Hascall & Hascall, 1981; Ruoslahti, 1988; Heinegard & Oldberg, 1989) or a limited number of core protein molecules, as reported for proteoglycans from brain (Oohira et al., 1988). Digestion with chondroitinase indicates that the heterogeneity in size of the sea cucumber proteoglycans lies with the core proteins (Figures 6 and 8).

Incubation of the high-molecular-weight fraction with trypsin forms proteoglycans with the same electrophoretic mobility as the smaller size fractions (Figure 9). Thus, it may be that the various fractions of sea cucumber proteoglycans arise from in vivo partial protease cleavage of a high-molecular-weight precursor (*trypsin-sensitive region*, Figure 10). It is unlikely that the sea cucumber proteoglycans are cleaved by proteases during the extraction or purification procedures, since the relative proportions of the various fractions remain the same throughout (Figure 4). In addition, a mixture of protease inhibitors were used during the entire extraction and purification steps.

Connective tissues of invertebrates and vertebrates have been compared in their macromolecular structures and functional properties (Mathews, 1975). Echinoderms have a catch connective tissue, which can rapidly and reversibly alter its mechanical properties. These alterations, which are thought to be neuronally controlled, allow the tissue to change its length by more than 200% (Trotter & Koob, 1989). The connective tissue of echinoderms contains parallel collagen fibrils, which are able to slide past one another during length changes but are inhibited from sliding when the tissue is in "catch". It has been a puzzling question of how a tissue composed of oriented collagen fibrils flows like a fluid and then becomes rigid. Since proteoglycans are associated with the surface of collagen fibrils (Junqueira et al., 1980), it has been suggested that proteoglycans may be an important component of the stress-transfer matrix in echinoderms (Trotter & Koob, 1989). Perhaps the presence of proteoglycans with a wide variety of sizes is required to meet these different functional demands of the catch connective tissue of sea cucumber.

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