Purification and Characterization of Protease-resistant Secretory Granule Proteoglycans Containing Chondroitin Sulfate Di-B and Heparin-like Glycosaminoglycans from Rat Basophilic Leukemia Cells*

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Proteoglycans were extracted from nuclease-digested sonicates of 10⁷ rat basophilic leukemia (RBL-1) cells by the addition of 0.1% w/t zwittergent 3-12 and 4 M guanidine hydrochloride and were purified by sequential CsCl density gradient ultracentrifugation, DE52 ion exchange chromatography, and Sepharose CL-6B gel filtration chromatography under dissociative conditions. Between 0.3 and 0.8 mg of purified proteoglycan was obtained from approximately 1 g initial dry weight of cells with a purification of 200-800-fold. The purified proteoglycans had a hydrodynamic size of 10⁴, 100,000-150,000, and were resistant to degradation by a molar excess of trypsin, \( \alpha \)-chymotrypsin, Pronase, papaain, chymopapain, collagenase, and elastase. Amino acid analysis of the peptide core revealed a preponderance of Gly (35.4%), Ser (22.5%), and Ala (9.5%).

Approximately 70% of the glycosaminoglycan side chains of RBL-1 proteoglycans were digested by chondroitinase ABC and 27% were hydrolyzed by treatment with nitrous acid. Sephadex G-200 chromatography of glycosaminoglycans liberated from the intact molecule by \( \beta \)-elimination demonstrated that both the nitrous acid-resistant (chondroitin sulfate) and the chondroitin ABC-resistant (heparin/heparan sulfate) glycosaminoglycans were of approximately 10⁴, 12,000. Analysis of the chondroitin sulfate disaccharides in different preparations by amino-cyano high performance liquid chromatography revealed that 9-29% were the unusual disulfated disaccharide chondroitin sulfate di-B (IdUA-2-SO₄⁺GalNAc-4-SO₄); the remainder were the monosulfated disaccharide GlcUA⁺GalNAc-4- SO₄. Subpopulations of proteoglycans in one preparation were separated by anion exchange high performance liquid chromatography and were found to contain chondroitin sulfate glycosaminoglycans whose disulfated disaccharides ranged from 9-49%. However, no segregation of subpopulations without both chondroitin sulfate di-B and heparin/heparan sulfate glycosaminoglycans was achieved, suggesting that RBL-1 proteoglycans might be hybrids containing both classes of glycosaminoglycans. Sepharose CL-6B chromatography of RBL-1 proteoglycans digested with chondroitinase ABC revealed that less than 7% of the molecules in the digest chromatographed with the hydrodynamic size of undigested proteoglycans, suggesting that at most 7% of the proteoglycans lack chondroitin sulfate glycosaminoglycans.

RBL-1 cells stimulated with the calcium ionophore A23187 exocytosed proteoglycans, histamine, and \( \beta \)-hexosaminidase in comparable noncytotoxic dose-related fashion, and regression analyses of the net per-centsages released indicated that proteoglycans were in secretory granules. The exocytosed and retained proteoglycans were of similar size, buoyant density, and glycosaminoglycan composition, providing further evidence for a single pool of secretory granule proteoglycans. The co-purification and co-localization of proteoglycans containing heparin/heparan sulfate glycosaminoglycans and chondroitin sulfate di-B glycosaminoglycans introduces the possibility that this tumor cell polymerizes both classes of glycosaminoglycans onto a single peptide core. The intragranular proteoglycans of the RBL-1 cell, whether two separate classes or hybrid molecules, are distinguished from those of the rat serosal mast cell in being one-fifth the hydrodynamic size and having predominantly chondroitin sulfate di-B glycosaminoglycans. However, intragranular proteoglycans of RBL-1 cells and rat serosal mast cells possess the common properties of oversulfation, protease resistance, and a preponderance of Gly and Ser in their peptide cores.

The RBL-1 cell line was established from a chemically generated rat basophilic leukemia (1). Its morphology varies with the rate of cell division, and it can resemble an undifferentiated sparsely granulated promyelocyte or a well-granulated mature basophil (2). The RBL-1 cell contains 0.1-1.0 pg/cell of histamine in secretory granules which can be released in some sublines (3) by cross-linking of its well-characterized surface IgE receptors (4-10). All lines are amenable

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to activation by the calcium ionophore A23187 under the appropriate conditions, resulting in de novo generation and release of prostaglandins (11) and leukotrienes (12-14), as well as release of preformed mediators (15).

Rat (16-19) and mouse (20) serosal mast cells contain an extremely acidic intragranular heparin proteoglycan of Mr, 0.75-1 x 10^6. Heparin proteoglycan is distinguished from extracellular matrix proteoglycans by having regions of glycosaminoglycans rich in an unusual trisulfated disaccharide, IdUA-2-SO4-+GlcN,6-diSO4, by being resistant to proteolysis and by having a unique peptide core which is primarily a copolymer of Ser and Gly (18, 19). The intragranular proteoglycan of the mouse interleukin 3-dependent mast cell derived from bone marrow (20, 21), fetal liver, and immune lymph node (22) is a chondroitin sulfate proteoglycan of about Mr, 250,000 possessing an unusual disulfated disaccharide, GlcUA-GalNAc-4,6-diSO4, termed chondroitin sulfate E, as a major glycosaminoglycan constituent.

The recognition of an oversulfated chondroitin proteoglycan in the secretory granule of the cultured mouse mast cell subclass suggested that other histamine-containing cells might contain the same or a homologous oversulfated non-heparin proteoglycan. RBL-1 cells maintained in vitro from bone marrow (20, 21), fetal liver, and immune lymph node (22) is a chondroitin sulfate proteoglycan of about Mr, 250,000 possessing an unusual disulfated disaccharide, GlcUA-GalNAc-4,6-diSO4, termed chondroitin sulfate E, as a major glycosaminoglycan constituent. In the present study, proteoglycans were obtained from the RBL-1 cell line maintained in tissue culture and purified to apparent homogeneity by ultracentrifugation and conventional chromatography techniques performed under dissociative conditions. The hydrodynamic size of the purified proteoglycans and glycosaminoglycans, the disaccharide composition of the glycosaminoglycans, and the amino acid composition of the purified proteoglycans were determined. Ionomophore-induced secretion experiments demonstrated an intragranular localization of these proteoglycans. The RBL-1 proteoglycans share properties with rat serosal mast cell heparin proteoglycans of oversulfation and protease resistance, suggesting that these molecules are members of a class of intragranular proteoglycans that fulfill related functional requirements; however, each population of proteoglycans has a distinct physicochemical composition characteristic of the particular cell type.

MATERIALS AND METHODS

Cell Culture and Radiolabeling—Adherent RBL-1 cells were maintained in 175-cm^2 flasks containing 80 ml of Earle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (Grand Island Biological Co., Grand Island, NY) at pH 7.2 in a humidified 37 °C incubator with a 6% CO2 atmosphere. The medium was changed twice per week until the cells reached confluence (0.5-1 x 10^6 cells/flask). Cells were passaged by washing with Hanks' balanced salt solution without calcium or magnesium, incubating with 10 ml of a trypsin-EDTA solution (Gibco) for 10 min at 37 °C, and dividing the detached cells into 10 flasks and 100 μl/ml streptomycin (Grand Island Biological Co., Grand Island, NY) at pH 7.2 in a humidified 37 °C incubator with a 6% CO2 atmosphere. The medium was changed twice per week until the cells reached confluence (0.5-1 x 10^6 cells/flask). Cells were passaged by washing with Hanks' balanced salt solution without calcium or magnesium, incubating with 10 ml of a trypsin-EDTA solution (Gibco) for 10 min at 37 °C, and dividing the detached cells into 10 flasks with fresh medium. To radiolabel proteoglycans biosynthetically for subsequent purification and analysis, confluent cells were incubated with 100 μCi of [35S]sulfate (New England Nuclear)/ml of medium for 4 h at 37 °C. Ten flasks (approximately 10^6 cells) were harvested, washed, sedimented at 400 x g, resuspended in 1 ml of 0.05 M sodium acetate, pH 6.0, and disrupted with 30 pulses of a Branson sonifier (Danbury, CT). In the standard procedure, intact nucleic acids, which were found to copurify with RBL-1 proteoglycans, were degraded by incubation of the cell sonicate with 100 units/ml ribonuclease A and 1000 units/ml deoxyribonuclease (Sigma) for 30 min at 37 °C, after which MgSO4 was added to a final concentration of 5 mM for a further 30-min incubation. The digestion mixture was then suspended in 0.1 ml of 0.05 M sodium acetate containing 1% (w/v) Zwittergent 3-12 detergent (Calbiochem-Behring), 0.1 M 6-aminohexanoic acid, 0.1 M sodium EDTA, 5.0 mM benzamidine HCl, and 1.0 mM sodium iodoacetamide, followed by 0.9 ml of 4 M GnHCl in the same buffer. In order to determine the rate of incorporation of [35S]sulfate into macromolecules, a sample of this nuclelease-treated cell extract was diluted to 0.5 ml in 0.1 M Tris-HCl, 0.1 M sodium sulfate, 4 M GnHCl, pH 7.0 (TSG buffer) and chromatographed on a PD-10 gel filtration column (Pharmacia Fine Chemicals) equilibrated in the same buffer. The proteoglycans (0.5 ml) were dialyzed and mixed with 10 ml of 10 M HCl, 10 ml of hydrofluor (National Diagnostics, Somerville, NJ), and the radioactivity eluting in the void volume of the PD-10 column was quantitated by β-scintillation counting. To minimize the possibility of proteolytic degradation of the proteoglycans, in one preparation the nuclelease digestion was omitted, the cell pellet was directly suspended in acetate buffer with detergent and inhibitors followed by 4 M GnHCl, and the extracted proteoglycans were isolated, purified, and analyzed as described below for proteoglycans extracted in the standard way from nuclelease-treated cell sonicates.

Proteoglycan Purification—Extracts of 10^6 RBL-1 cells were subjected to CsCl density gradient ultracentrifugation under dissociative conditions by adding CsCl in 0.05 M Tris-HCl, 4 M GnHCl, pH 7.0, to a starting density of 1.4 g/ml and centrifuging at 95,000 x g for 48 h at 17 °C. The centrifuged samples were frozen at -70 °C and cut into two equal fractions; the bottom half with the greater buoyant density was termed D1 and the top half, D2. Twenty-five microliter portions of each fraction were applied to PD-10 columns and eluted with TSG buffer. Fraction D1, which contained the majority of the 35S-macromolecules, was dialyzed for 3 days against 0.1 M ammonium bicarbonate, lyophilized, resuspended in 2 ml of 0.05 M Tris-HCl, 4 M GnHCl, pH 7.25 (TU buffer), and 100 μg/ml streptomycin, containing DE-52 cellulose (Whatman) equilibrated in the same buffer. The resin was washed with 20 ml of buffer and eluted with an 80-ml linear gradient to 1 M NaCl in the same buffer, and 1 ml fractions were collected. The absorbance at 280 nm and the conductivity of the fractions were measured, and the radioactivity in 25 μl portions determined. Fractions containing 35S-macromolecules were pooled, dialyzed against 0.1 M ammonium bicarbonate, lyophilized, resuspended in 200 μl of TSG buffer, and applied to a 0.6 x 80-cm Sepharose CL-6B column. The column was eluted with TSG buffer by gravity flow at a rate of 2.5 ml/h, and 60 0.5-ml fractions were collected. The absorbance at 280 nm was measured, and the radioactivity in 20 μl of each of the fractions was determined. The hydrodynamic size of the RBL-1 proteoglycans was estimated by calculating the Kav and comparing it with values of proteoglycans of known hydrodynamic size chromatographed on Sepharose CL-6B. Fractions containing 35S-proteoglycans were pooled, dialyzed against 0.1 M ammonium bicarbonate and, lyophilized. The purification factor was calculated by comparing the ratio of 35S-macromolecules/dry weight of material, expressed as cpm/mg, in the starting material and in the purified proteoglycan preparations.

Protease Susceptibility of RBL-1 Proteoglycans—The susceptibility of RBL-1 proteoglycans to proteolytic degradation was evaluated using tunicamycin (204 units/ml ribonuclease A, 100 units/ml trypsin), a-chymotrypsin (47 units/mg), papain (21 units/mg), chymopapain (4.5 units/mg), elastase (34 units/mg) (Sigma), and Pronase (77 units/mg) (Calbiochem-Behring). Approximately 1 μg of purified proteoglycans (5000 cpm) was incubated with 5 μg of each of these enzymes in Hanks' balanced salt solution with calcium and magnesium (except papain, which was incubated in Hanks' balanced salt solution with 0.01 M EDTA and 0.05 M cysteine) at a final volume of 100 μl for 1 h at 37 °C; 100 μl of TSG buffer was then added and the mixture was applied to the Sepharose CL-6B column. The elution profile of 35S-proteoglycans after incubation with each protease was compared with that of a prestained control proteoglycan standard from Syrian hamster chondrosarcoma chondrocytes (24) digested in parallel and chromatographed on a Sepharose CL-4B column to evaluate the proteolytic susceptibility of matrix proteoglycans.

Disaccharide Composition and Hydrodynamic Size of the Glycosaminoglycans of RBL-1 Proteoglycans—The composition of the glycosaminoglycans of the purified RBL-1 proteoglycans was determined by chemical and enzymatic degradation. The proportion of heparan and/or heparin sulfate was evaluated by susceptibility to nitrous acid treatment (20, 25, 26). One hundred μg of heparin carrier (Sigma) was mixed with 10,000 cpm of purified RBL-1 proteoglycans in a volume of 100 μl. Dimethyl butynithane (100 μl) and butyl nitrite (10 μl) were added, and the reaction was allowed to proceed at -20 °C for 16 h. As a control, 10,000 cpm of [3H]heparin (New England Nuclear) was incubated in parallel. Both reactions were halted by the addition of 7.5 μl of a saturated solution of sodium acetate and 250 μl of TSG...
buffer. Hydrolysates were chromatographed on PD-10 columns in 4 M HC1 to assess the degradation of glycosaminoglycans. The proportion of 35S-glycosaminoglycans that was chondroitin sulfate was determined by digestion of purified RBL-1 proteoglycans with chondroitinase ABC according to the procedure of Saito et al. (27). Radiolabeled proteoglycan (50,000 cpm) and 100 ng each of chondroitin sulfate A and C carriers were incubated with 0.4 unit of chondroitinase ABC (Miles Laboratories, Inc., Elkhart, IN) in the presence of 0.0 M sodium fluoride to inhibit contaminating sulfatases (28) for 1 h at 37 °C. The percentage of glycosaminoglycans digested to unsaturated disaccharides was assessed by PD-10 chromatography of the reaction mixtures. The proportion of those 35S-disaccharides that contained iduronic rather than glucuronic acid, i.e. those that were uronic sulfate-like, was estimated by subtraction of the percentage of disaccharides generated by chondroitinase AC digestion, following the same protocol, from the percentage obtained by digestion with chondroitinase ABC.

The composition of the chondroitin sulfate glycosaminoglycans was further analyzed by amino-cyano HPLC (29). 35S-Disaccharides liberated by chondroitinase ABC digestion were separated from under-degraded proteoglycans, enzyme, and contaminating macromolecules by an 85% ethanol extraction in which the digestion mixture was diluted to 5 volumes of ethanol. The ethanol was cooled to -20 °C for 1 h and centrifuged in a Beckman Microfuge at 8,000 × g for 5 min. The supernatant was decanted, dried over nitrogen, and resuspended in the HPLC solvent, which was 70% acetonitrile/methanol (31, v/v) and 30% 0.5 M ammonium acetate/acetate acid, pH 5.3, with an approriately matched Chromatography of the disaccharides (Woburn, MA) gradient HPLC system controlled by an Apple IIe computer (Cupertino, CA). A 4.6 × 250-mm Partisil-10 PAC amino-cyano-substituted normal phase silica column, with a 4.6 × 25-mm precolumn containing the same packing (Whatman) was used for separating disaccharides. The ultraviolet absorbance of the column effluent was monitored continuously at 232 nm, the absorbance maximum for unsaturated disaccharides, with a spectroMonitor D (LDC/Milton Roy, Riviera Beach, FL). Peak analysis was performed by the Apple IIe in conjunction with a Gilson (Villiers le Bel, France) Data Master and accompanying software. Eluates containing radiolabeled disaccharides were collected for 0.5-min intervals and quantitated by β-scintillation counting in Hydrofluor. One-μg portions of ΔDi-OS, ΔDi-6S, and ΔDi-4S (Miles Laboratories, Inc.) were routinely used as calibration standards, and disaccharides generated from the following glycosaminoglycans were used as reference polylsulfated disaccharides: chondroitin sulfate di-B (the unsaturated disaccharide generated by chondroitinase ABC is ADi-diSB) from squid cranial cartilage (34, 35) or from mouse bone (32). The retention times of these disaccharides were: ΔDi-OS, 5 min; ΔDi-6S, 6 min; ΔDi-4S, 7 min; ΔDi-diSB, 10 min; ΔDi-diSβ, 14.5 min; ΔDi-diSα, 16.5 min; and ΔDi-tris, 21 min.

Glycosaminoglycans were liberated from 60,000 cpm of purified RBL-1 proteoglycans by β-elimination in 30 μl of 0.5 M NaOH for 16 h at 4 °C followed by neutralization with 30 μl of 0.5 M acetic acid. This glycosaminoglycan preparation was divided into three equal portions: one was untreated; one was subjected to the nitrous acid degradation of glycosaminoglycans intact; and one was subjected to sonication for 30 s, dialyzed for 2 days against 0.1 M ammonium bicarbonate, lyophilized, and resuspended in 1.4 g/ml CaCl2 in 0.05 M Tris-HCl, 4 mM NaHCO3 pH 7.0, for density gradient ultracentrifugation. The ultracentrifuged samples were divided into two buoyant density fractions, D1 and D2, and samples of each fraction were subjected to gel chromatography to quantify 35S-macromolecules. The D1 fractions, which contained the majority of the 35S-macromolecules from both the supernatants and pellets, were dialyzed against 0.1 M ammonium bicarbonate for 3 days and lyophilized. These partially purified proteoglycan samples were used to assess the hydrodynamic size and glycosaminoglycan composition of the ADi-OS and ADi-Sβ proteoglycans, and to determine the presence of contaminating macromolecules. The ADi-OS and ADi-Sβ proteoglycans were eluted with a linear gradient from 0.5-1 M NaCl for 4 h at 37 °C. TSG buffer was added and the mixture was applied to the Sepharose CL-6B column and eluted with TSG buffer as described. A control containing 25,000 cpm of undisaggregated 35S-proteoglycans and carriers was also chromatographed.

Gel Filtration of Chondroitinase ABC-digested RBL-1 Proteoglycans—Purified 35S-proteoglycans (25,000 cpm) and 100 ng each of chondroitin sulfate A and C carriers were incubated with 0.4 unit of chondroitinase ABC in the presence of 0.01 M sodium fluoride for 1 h at 37 °C. TSG buffer was added and the mixture was applied to the Sepharose CL-6B column and eluted with TSG buffer as described. A control containing 25,000 cpm of undisaggregated 35S-proteoglycans and carriers was also chromatographed.

Anion Exchange HPLC of Purified RBL-1 Proteoglycans—The Rabin/Apple Ile gradient HPLC system was used to perform anion exchange HPLC of purified RBL-1 proteoglycans. A 4.6 × 25-cm Aquapore AX-100 column with a 3-cm guard column of the same packing (Brownlee Labs, Inc., Santa Clara, CA) was equilibrated with 200 ml of TUF buffer containing 0.5 μM NaCl at a flow rate of 1 ml/min, which resulted in pump pressures of approximately 550 p.s.i. Five thousand cpm of purified RBL-1 proteoglycans (approximately 1 μg) dissolved in the same buffer was injected onto the column, which was washed for 20 min at the same flow rate. Proteoglycans were eluted with a linear gradient from 0.5-1 M NaCl in 50 min, followed by a 20-min wash at 1 M NaCl in TUF buffer. The ultraviolet absorbance of the column eluate was monitored continuously at 280 nm on the spectroscope D. Fractions were collected for 1-min intervals and analyzed for radioactivity in Hydrofluor. One-μg portions of ADi-OS, ADi-diSβ, and ADi-diSα were added to TUF buffer and dialyzed against samples of each fraction was determined by β-scintillation counting. The fractions containing radioactive activity were pooled into three portions, based on increasing retention times of the column. The glycosaminoglycan content of each pool was determined by nitrous acid degradation and chondroitinase ABC digestion followed by amino-cyano HPLC disaccharide analysis of replicate samples.

Amino Acid Composition of the Peptide Core—Two samples of purified RBL-1 proteoglycans were used for analysis of the amino acid composition of the core peptide material. One mg of each sample of RBL-1 proteoglycans was subjected to acid hydrolysis in boiling HCl for 24 h and analyzed for amino acid content on a Beckman analyzer.
model 119CLW/126 amino acid analyzer. Integrated areas of optical absorbance were compared with areas obtained from standard amino acids to quantify relative amounts in the samples, which were converted to per cent of total amino acids.

RESULTS

Biosynthetic Labeling and Purification of Proteoglycans—RBL-1 cells maintained in tissue culture incorporated [³⁵S] sulfate into macromolecules at a rate of 2800 ± 1650 cpm/10⁶ cells/h (mean ± S.D., n = 5). When nuclease-treated cell extracts were ultracentrifuged in CsCl at a starting density of 1.4 g/ml under dissociative conditions, 70.4 ± 12.8% (mean ± S.D., n = 8) of the total cell-associated [³⁵S]-macromolecules were recovered in the D1 fraction of the gradient. Because most protein, carbohydrate, and lipid contaminants are of lesser buoyant density than proteoglycans and appear in fraction D2, that fraction was discarded.

Fraction D1 was dialyzed, lyophilized, and subjected to DE52 ion exchange chromatography. All of the [³⁵S]-macromolecules eluted in a single sharp peak at a conductivity of 30-42 millisiemens, approximately equal to 0.4-0.65 M NaCl in this buffer (Fig. 1). Material with significant ultraviolet absorbance eluted from the column in a slightly earlier peak, overlapping with the [³⁵S]-proteoglycans. The fractions containing radioactivity were pooled, dialyzed, lyophilized, and chromatographed on Sepharose CL-6B. [³⁵S]-Macromolecules filtered as a single sharp peak with a $K_v$ of 0.25 (Fig. 2), indicating a hydrodynamic size of approximately $M_v$ 100,000-150,000. Proteoglycans purified by this procedure from RBL-1 cell sonicates that were not treated with nucleases had the same buoyant density, charge characteristics, and hydrodynamic size; however, material that was presumed to be nucleic acid because of its ultraviolet absorbance ratio for 256 nm/280 nm of 1.4 coeluted with proteoglycans upon gel filtration. The nuclease procedure cleaved the nucleic acids to oligonucleotides, resulting in a shift of $K_v$ from 0.25 to 0.9, well separated from proteoglycans (Fig. 2). The [³⁵S]-macromolecules obtained after Sepharose CL-6B chromatography lacked material which could be detected by the Lowry assay (41) and were, therefore, deficient in aromatic amino acid-containing protein. These pooled [³⁵S]-macromolecules were considered to be purified proteoglycans. The dry weight of purified proteoglycans obtained from approximately 1 g dry weight ($lo^9$) cells ranged from 0.3-0.8 mg in four preparations, and purifications were calculated to be from 200-800-fold.

Protease Resistance of Purified RBL-1 Proteoglycans—Replicate samples of approximately 1 µg of purified [³⁵S]-proteoglycans were incubated separately with an excess of each of seven proteases under appropriate conditions of pH and cation concentrations, and the individual digests were chromatographed on Sepharose CL-6B. The elution patterns of an undigested control sample and a sample treated with a molar excess of Pronase for 1 h were identical (Fig. 3A), indicating...
that there was no detectable decrease in the hydrodynamic size of the RBL-1 proteoglycans. None of the enzymatic treatments resulted in an alteration in this elution profile. In contrast, [35S]sulfate-labeled chondroitin sulfate proteoglycans from the Swarm rat chondrosarcoma chondrocyte, which treatments resulted in an alteration in this elution profile. In contrast, [35S]sulfate-labeled chondroitin sulfate proteoglycans from the Swarm rat chondrosarcoma chondrocyte, which chromogaphed in the void volume of a Sepharose CL-4B column, were appreciably decreased in hydrodynamic size by digestion with Pronase (Fig. 3B) and with each of the other proteases when treated as described.

Glycosaminoglycan Side Chains of RBL-1 Proteoglycans—Purified RBL-1 proteoglycans were enzymatically digested with chondroitinase ABC or AC or were hydrolyzed with nitrous acid, and the percentage of disaccharides liberated by each procedure was quantitated by PD-10 chromatography. In the untreated control sample of 35S-proteoglycans all radioactivity filtered in the void volume of a PD-10 column (Fig. 4A). Treatment with chondroitinase ABC resulted in 64% of the radioactivity being associated with disaccharides eluting in the included column volume (Fig. 4B), whereas treatment with chondroitinase AC liberated 45% of the radioactivity as disaccharides (Fig. 4C). Thirty-six per cent of the 35S-proteoglycans were hydrolyzed to oligosaccharides by nitrous acid, and the percentage of disaccharides liberated by nitrous acid treatment (Fig. 4D). These findings indicate that 64% of the glycosaminoglycans present in this preparation of proteoglycans were chondroitin sulfates of which 19% contained iduronic acid, whereas 36% were either heparin or heparan sulfate. Six different preparations of purified RBL-1 proteoglycans yielded material that was 71 ± 9% (mean ± S.D.) digested by chondroitinase ABC and 27 ± 12% hydrolyzed by nitrous acid.

The chondroitinase ABC-generated unsaturated disaccharides were analyzed by amino-cyano HPLC. 35S-Disaccharides eluted in a major peak at a retention time of 7 min, corresponding to ΔDi-4S, and a second peak at 14 min, corresponding to ΔDi-diS6 (Fig. 5). Chondroitinase ABC digests of five different samples of purified RBL-1 proteoglycans revealed that 12 ± 0.3% (mean ± S.D.) of the disaccharides coeluted with the ΔDi-diS6 standard. Since digestion with chondroitinase AC generated ΔDi-4S only, the disaccharide which eluted at the retention time of ΔDi-diS6 contained iduronic acid and was presumed to be identical to the chondroitin sulfate di-B disaccharide from other sources (29–31), and not the glucuronic acid-containing isomer.

The glycosaminoglycans liberated from whole RBL-1 proteoglycans by β-elimination chromatographed with a single broad peak of 0.4 ± 0.05 (k50) on Sephadex G-200, indicating a M of 12,000 (Fig. 6). The chondroitin sulfate glycosaminoglycans, which remained intact after nitrous acid hydrolysis of the total glycosaminoglycans, and the heparin/heparan sulfate glycosaminoglycans, which remained intact after chondroitinase ABC digestion, had hydrodynamic sizes not significantly different from each other or from the untreated glycosaminoglycans (Fig. 6).

Anion Exchange HPLC of RBL-1 Proteoglycans—When 5000 cpm (approximately 1 µg) of purified RBL-1 proteoglycan was injected onto the AX-1000 anion exchange HPLC column, a broad peak containing 94% of the applied radioactivity was eluted (Fig. 7). No peak with ultraviolet absorbance at 280 nm was detected with the spectrophotometer set at a full scale sensitivity of 0.1 absorbance units. As indicated in the figure, the fractions containing radioactivity were divided into three portions, pools I, II, and III, in order of increasing retention times, and the glycosaminoglycans of the proteoglycans in each pool were analyzed by nitrous acid hydrolysis and chondroitinase ABC digestion coupled with amino-cyano HPLC.

More than 90% of the radiolabeled disaccharides and oligosaccharides in each pool were identified (Table I), and it was observed that anion exchange HPLC did not separate proteoglycans containing only chondroitin sulfate glycosaminoglycans from proteoglycans containing only heparin/heparan sulfate glycosaminoglycans. However, the percentages of chondroitin sulfate disaccharides which were disulfated were greater in the pools of proteoglycans eluting from the column with longer retention times, being 9% in pool I, 22% in pool II, and 49% in pool III. Twenty-nine per cent of the chondroitin sulfate disaccharides in the sample of purified proteoglycans injected onto the AX-1000 column were ΔDi-diS6.

Gel Filtration of Chondroitinase ABC-digested RBL-1 Proteoglycans—The Kav of a sample of purified 35S-proteoglycans mixed with 100 µg each of chondroitin sulfate A and C and rechromatographed on Sepharose CL-6B was 0.23 (Fig. 8). After incubation of a replicate mixture with chondroitinase...
FIG. 6. Sephadex G-200 gel filtration chromatography of glycosaminoglycans liberated from 60,000 cpmp of purified RBL-1 proteoglycans by β-elimination and subjected to no treatment (C—C); nitrous acid hydrolysis, leaving chondroitin sulfate glycosaminoglycans intact (Δ—Δ); or chondroitinase ABC digestion, leaving heparin/heparan sulfate glycosaminoglycans intact (O—O). Incomplete β-elimination left some 35S-proteoglycan intact, which appeared at the void volume (V₀). Disaccharides and oligosaccharides liberated by chemical or enzymatic treatment appeared near the total column volume (Vₜ).

![Graph showing gel filtration chromatography results](image)

FIG. 7. AX-1000 anion exchange HPLC of purified RBL-1 proteoglycans. Proteoglycans were injected onto the column in TGS buffer with 0.5 M NaCl and eluted with a 20 mM/min gradient from 0.5-1.0 M NaCl followed by a 20-min wash at 1 M NaCl. Column fractions (0.5 ml) were screened for radioactivity. The ultraviolet absorbance at 280 nm was monitored continuously but did not exceed baseline. The broad peak of 35S-proteoglycans was divided into three pools (I, II, and III) as indicated.

![Graph showing anion exchange HPLC results](image)

TABLE I

| Disaccharide structure  | Pool I | Pool II | Pool III |
|------------------------|--------|---------|----------|
| Heparin-like           | 15     | 19      |          |
| Chondroitin sulfate    | 75     | 74      | 75       |
| ΔDi-4S                 | 91     | 78      | 51       |
| ΔDi-diS₄              | 9      | 22      | 49       |

ABC for 1 h at 37 °C, 65.8% of the 35S radioactivity appeared near V₀ with a Kᵥ of 0.91, representing [35S]chondroitin sulfate disaccharides. A peak containing 27.4% of the radioactivity appeared at a Kᵥ of 0.43 and was presumed to be proteoglycan core peptide with heparin-like [35S]glycosaminoglycans attached. 6.7% of the radioactivity chromatographed with a Kᵥ of 0.23, suggesting that at most this percentage of the RBL-1 proteoglycans contained mainly heparin-like glycosaminoglycans which were not susceptible to chondroitinase ABC digestion and were not shifted in hydrodynamic size relative to the undigested control.

FIG. 8. Sepharose CL-6B gel filtration chromatography in TSG buffer of purified RBL-1 proteoglycans (C—C) and purified RBL-1 proteoglycans digested for 1 h at 37 °C with chondroitinase ABC (O—O). The radioactivity in the 0.25-ml fractions was determined.

![Graph showing gel filtration chromatography results](image)

**Exocytosis of Proteoglycans from RBL-1 Cells** — The net percent release of histamine, β-hexosaminidase, lactate dehydrogenase, and proteoglycans in response to increasing doses of calcium ionophore A23187 was determined (Fig. 9). The percentages of histamine, β-hexosaminidase, and 35S-macromolecules spontaneously released during the 15-min incubation period at 37 °C were 0.5 ± 0.4, 1.3 ± 0.4, and 4.3 ± 0.5% (mean ± S.D., n = 4), respectively. The addition of increasing doses of ionophore resulted in the concomitant exocytosis of histamine, β-hexosaminidase, and proteoglycans, with maximal release values of 31.6 ± 3.0, 23.6 ± 3.7, and 35.1 ± 3.2% (mean ± S.D., n = 4), respectively, at 20 μM ionophore. Release of the cytosolic marker lactate dehydrogenase was detected only at this highest ionophore dose, at which 3.4 ± 3.2% of this enzyme was released. Data from this and four similar dose response experiments were subjected to linear regression analysis of net per cent release of β-hexosaminidase versus histamine (Fig. 10A). For 25 data points, the line generated had a slope of 0.63 and a y intercept of 0.79%, with
the coefficient of determination, $r^2$, equal to 0.93. Linear regression analysis of four dose-response experiments (20 data points) generated a line with a slope of 0.60, a y intercept of 4.5%, and an $r^2$ of 0.72 for the net per cent release of $^{35}$S-proteoglycans versus histamine (Fig. 10B).

Activation of 175-cm$^2$ flasks of RBL-1 cells containing $^{35}$S-labeled proteoglycans with 10 $\mu$M calcium ionophore A23187 resulted in the exocytosis of 43% of the histamine, 31% of the $\beta$-hexosaminidase, and 20% of the $^{35}$S-proteoglycans without detectable release of lactate dehydrogenase. The secreted proteoglycans and the proteoglycans retained with the cells were partially purified in parallel by CsCl density gradient ultracentrifugation for subsequent determination of hydrodynamic size and glycosaminoglycan composition. Eighty-three per cent of the peptide core of the exocytosed proteoglycans and 81% of the cell-associated proteoglycans were recovered in the high density D1 fraction in the same ultracentrifuge run, and both populations of proteoglycans had $K_v$ values of 0.22 upon Sepharose CL-6B gel filtration chromatography. Analysis of the glycosaminoglycans of the exocytosed proteoglycans revealed that 32% were degraded by nitrous acid and 68% were susceptible to chondroitinase ABC; of the chondroitinase ABC-generated disaccharides, 15% were $\Delta$Di-diSB, 35% were $\Delta$Di-4S as analyzed by HPLC. The glycosaminoglycans of the retained proteoglycan were 8% susceptible to nitrous acid degradation and 80% digested by chondroitinase ABC; 12% of the chondroitin sulfate disaccharides were $\Delta$Di-diSB and 88% were $\Delta$Di-4S. In a second experiment in which 15% of the $^{35}$S-proteoglycans were released, 87% of the exocytosed and 63% of the retained proteoglycans were in the D1 fraction of the CsCl gradient. Both populations of proteoglycans had $K_v$ values of 0.2 upon Sepharose CL-6B chromatography. The exocytosed and retained proteoglycans had glycosaminoglycans that were 73 and 69% chondroitinase ABC susceptible and 69 and 85% were $\Delta$Di-diSB and 88% were $\Delta$Di-4S. In a second experiment in which 15% of the $^{35}$S-proteoglycans were released, 87% of the exocytosed and 63% of the retained proteoglycans were in the D1 fraction of the CsCl gradient. Both populations of proteoglycans had $K_v$ values of 0.2 upon Sepharose CL-6B chromatography. The exocytosed and retained proteoglycans had glycosaminoglycans that were 73 and 69% chondroitinase ABC susceptible and 12 and 14% nitrous acid susceptible, respectively. Nine per cent of the disaccharides from the chondroitin sulfate glycosaminoglycans of the exocytosed proteoglycans and 11% of the disaccharides from the glycosaminoglycans of the retained proteoglycans were $\Delta$Di-diSB. The most common amino acid was Gly (35.4%), followed by Ser (22.5%), Ala (9.5%), and Glx (6.6%).

**DISCUSSION**

Proteoglycans from RBL-1 cells maintained in vitro were purified and characterized to allow comparison with previously characterized protease-resistant Ser- and Gly-rich intragranular heparin proteoglycans of rat serosal and skin mast cells (16-19). Biosynthetically labeled proteoglycans were ex-
Proteoglycans have been found in a number of immuno-
and neurosecretory cells including mast cells, basophils (44),
eroehromaffin cells (45), and platelets (46). These cells
zyme cationic amines such as histamine, serotonin, or
catecholamines, as well as proteolytic and glycosidic enzymes.
The functions of the anionic proteoglycans that are packaged
into secretory granules with these mediators probably include
preventing diffusion of small amines by ionic retention, bind-
ing and intracellular inhibition of the large amounts of potent
degradiative enzymes, and pH and osmoregulation in the
granule. When the granule is exposed to the extracellular milieu,
proteoglycans may control the rate of solubilization of media-
tors into the tissue space. The high degree of sulfation and
the protease resistance of intragranular proteoglycans may be
important for preventing degradation in the granule microen-
vironment. In addition, these releasable proteoglycans have
been demonstrated to have important extracellular functions
of their own. Heparin inhibits the coagulation cascade in vivo
(47). Both heparin and chondroitin sulfate E glycosaminogly-
cans inhibit activation of the alternate complement pathway
(48, 49) and initiate the Hageman factor-dependent contact
activation pathway in vitro (50).

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