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Complexes of Heparin Proteoglycans, Chondroitin Sulfate E Proteoglycans, and [^{3}H]Diisopropyl Fluorophosphate-binding Proteins Are Exocytosed from Activated Mouse Bone Marrow-derived Mast Cells*

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The predominant [^{3}H]diisopropyl fluorophosphate (DFP)-binding proteins that are released from the secretory granules of activated mouse bone marrow-derived mast cells (BMMC) are demonstrated to have an isoelectric point of ~9.1 and to be complexed to proteoglycans. Upon Sepharose CL-2B chromatography of the supernatants of calcium ionophore-activated BMMC, 67–78% of the total exocytosed [^{3}H]DFP-binding proteins co-eluted in the excluded volume of the column as a >1 × 10^7 M_r complex bound to 4–7% of the total exocytosed proteoglycans. The remainder of the exocytosed proteoglycans, which filtered in the included volume of the gel filtration column with a K_{av} of 0.66, contained chondroitin sulfate E glycosaminoglycans. After dissociation of the large M_r complexes of [^{3}H]DFP-binding proteins-proteoglycans with 5 M NaCl and removal of the proteins via phenyl-Sepharose chromatography, the proteoglycans filtered from the Sepharose CL-2B column as a single peak with a K_{av} of 0.66. The susceptibility of 24–59% and 36–76% of the glycosaminoglycans in the large M_r complex to degradation by nitrous acid and chondroitinase ABC, respectively, indicated the presence of proteoglycans that contained heparin and chondroitin sulfate glycosaminoglycans. Disaccharide analysis revealed that the chondroitin sulfate in the high M_r complex was chondroitin sulfate E. Following chondroitinase ABC treatment of the large M_r complex, the residual heparin proteoglycans filtered on Sepharose CL-4B under dissociative conditions with the same K_{av} as the original, untreated proteoglycans. Thus, the protein-proteoglycan complexes that are exocytosed from activated mouse BMMC contain approximately equal amounts of proteoglycans of comparable size that bear either predominantly heparin or predominantly chondroitin sulfate E glycosaminoglycans. The demonstration of these secreted complexes indicates that the intragranular protease-resistant heparin and chondroitin sulfate E proteoglycans in the T cell factor-dependent BMMC bind serine proteases throughout the activation-secretion response.

The proteoglycans that reside in the secretory granules of rat (1–4) and mouse (5, 6) mast cells are more resistant to proteolytic degradation (1, 2, 7) and are more highly sulfated than the proteoglycans that reside on the plasma membranes of cells or in the extracellular matrices (8). Whereas ~750,000 M_r heparin proteoglycans are present in mouse (5, 6) and rat (1, 2) serosal mast cells, ~150,000 M_r chondroitin sulfate di-B proteoglycans occur in rat mucosal mast cells (8), and ~200,000 M_r chondroitin sulfate E proteoglycans are present in the secretory granules of mouse interleukin 3-dependent mast cells derived from either bone marrow (BMMC[1]), fetal liver, or lymph node progenitors (6, 9). In addition to proteoglycans, the secretory granules of all populations of mast cells studied to date contain 25,000–31,000 M_r serine proteases that are enzymatically active at neutral pH. The major neutral serine protease of the rat serosal mast cell, chymase, has a pI = 9.3 (10). This cationic protease is ionically bound to the negatively charged heparin proteoglycans when both are exocytosed from immunologically activated cells (11, 12). The activity of chymase against large, but not small, M_r substrates is substantially inhibited when it is bound to heparin proteoglycan (11, 13).

As assessed by [^{3}H]diisopropyl fluorophosphate (DFP) binding and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), mouse BMMC have in their granules DFP-binding proteins that range in M_r from 27,000 to 31,000 (14). At least one of the DFP-binding proteins secreted from activated BMMC is a serine protease, since human fibronectin is rapidly degraded at 37 °C at a substrate/protein ratio of ~200:1 by a DFP-inhibitable enzyme. In the current study, the pI of the predominant intragranular [^{3}H]DFP-binding proteins of the mouse BMMC is shown to be ~9.1. Upon activation of these mast cells, the majority of the cationic proteins in the secretory granules are exocytosed as large M_r complexes that contain approximately equal amounts of a chondroitin sulfate E proteoglycan and a previously unappreciated heparin proteoglycan.

EXPERIMENTAL PROCEDURES

Cell Culture, ^{35}S-Labeling, and Calcium Ionophore Activation of BMMC—Bone marrow cells from BALB/c mice (The Jackson Lab-

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[1] The abbreviations used are: BMMC, bone marrow-derived mast cells; DFP, diisopropyl fluorophosphate; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

oratory, Bar Harbor, ME) were cultured for 3–6 weeks in 50% enriched medium and 50% (v/v) WEHI-3 (American Type Culture Collection, Rockville, MD) conditioned medium as previously described (15). For experiments requiring ^{35}S-labeled proteoglycans, BMMC were incubated at a concentration of 5×10^6 cells/ml at 37 °C for 4–6 h in fresh medium containing 100 μCi/ml [^{35}S]sulfate (4000 Ci/mmol, New England Nuclear). Unlabeled or ^{35}S-labeled BMMC were washed twice in modified Tyrode's buffer containing 0.32 mM Ca^{2+}, 0.2 mM Mg^{2+}, and 0.5 g/liter gelatin (bovine skin type III, Sigma), washed once in modified Tyrode's buffer without gelatin, and resuspended at a concentration of 2×10^7 cells/ml in Tyrode's buffer without gelatin. After prewarming BMMC for 10 min at 37 °C, an equal volume of 1.0 μM calcium ionophore A23187 (Behring Diagnostics) in prewarmed gelatin-free modified Tyrode's buffer was added (16). Activation of the BMMC was allowed to proceed at 37 °C for 20 min. The cells were sedimented at 150 × *g* for 10 min at room temperature, the cell pellets were separated from the supernatants, and all samples were placed on ice for subsequent analysis. Cell viability was routinely monitored by following the cellular exclusion of trypan blue and the release of the cytosol marker lactate dehydrogenase (17). The granule marker β-hexosaminidase was measured as described (18). Release of cellular constituents was calculated by the formula: release = $S/(S + P)$, where S and P are the constituent contents of the supernatant and cell pellet, respectively.

Gel Filtration Chromatography and Analysis of the Glycosaminoglycan Composition of the Proteoglycans Exocytosed from Activated BMMC—In order to resolve the exocytosed proteoglycans by differences in hydrodynamic size, 1–3 ml of supernatants from $1–3 \times 10^7$ calcium ionophore-activated ^{35}S-labeled BMMC were applied at 4 °C to an 80 × 0.8-cm Sepharose CL-2B (Pharmacia P-L Biochemicals) column that had previously been equilibrated with 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.2; 0.5-ml fractions were collected. Blue dextran 2000 (Pharmacia) and [^{35}S]sulfate were used to determine the void and total volumes of the column, respectively. The ^{35}S-labeled proteoglycans in the collected fractions were quantified by β-scintillation counting of 50-μl samples, and the fractions containing ^{35}S-labeled proteoglycans were pooled by peaks. 100 μg each of chondroitin sulfate A (Miles Laboratories, Elkhart, IN) and heparin (porcine intestine, Sigma) were added as carriers to prevent losses, and the pooled fractions were dialyzed for 48 h against 0.1 M NH_4HCO_3, lyophilized, and redissolved in 0.5 ml of distilled water for further analysis. In one experiment, the gel filtration profile of the unlabeled proteoglycans exocytosed from BMMC was determined by quantifying the uronic acid in each fraction by the carbazole reaction method (19). In addition, a pulse-chase experiment was performed to determine if the hydrodynamic size of the ^{35}S-labeled proteoglycans changed with time. Mouse BMMC were incubated with [^{35}S]sulfate for 4 h, washed in ^{35}S-free conditioned medium, and cultured in ^{35}S-free conditioned medium for 24 h prior to activation with calcium ionophore and subsequent Sepharose CL-2B column chromatography of the supernatants.

To determine the effect on the hydrodynamic size of removing chondroitin sulfate glycosaminoglycan side chains from the proteoglycans derived from the high M_r complex, 250-μl samples were treated with chondroitinase ABC as described below or with buffer alone. The reactions were terminated by adding an equal volume of 8 M guanidine-HCl, and the samples were applied to an 85 × 0.8-cm column of Sepharose CL-4B that had previously been equilibrated with 20 μg/ml of heparin and the dissociative buffer of 4 M guanidine-HCl, 0.1 M sodium sulfate, and 0.1 M Tris-HCl, pH 7.2. 1-ml fractions were collected and mixed with 1 ml of ethanol and 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ), and then quantified for radioactivity.

The glycosaminoglycan composition of the exocytosed proteoglycans present in the different pooled fractions from Sepharose CL-2B gel filtration was determined by both chemical and enzymatic degradative techniques. The proportion of ^{35}S-labeled heparin and/or heparan sulfate was ascertained by determining the extent of the susceptibility of the ^{35}S-labeled proteoglycans to nitrous acid degradation (20). ^{35}S-Labeled proteoglycan samples (800–3000 cpm) were suspended on ice in 100 μl of water containing 100 μg of heparin carrier. Dimethoxyethane (150 μl) and butyl nitrite (30 μl) were added, and the reactions were allowed to proceed at −20 °C for 24 h. As a control, 1 μCi of ^{3}H-labeled heparin (New England Nuclear) was treated in parallel. The reactions were terminated by the addition of 50 μl of saturated sodium acetate and 300 μl of 4 M guanidine-HCl. The acid hydrolysates were analyzed by Sephadex G-25/PD-10 (Pharmacia) chromatography using 4 M guanidine-HCl, 0.1 M sodium sulfate, and 0.1 M Tris-HCl, pH 7.0, as the equilibration and elution buffer. Each 0.5-ml fraction was mixed with 1 ml of ethanol and 10 ml of Hydrofluor, and the radioactivity was measured by β-scintillation counting. Degradation of the ^{35}S-labeled proteoglycans was quantified by determining the shift of ^{35}S-radioactivity from the void volume to the included volume of the column. The extent of susceptibility of the ^{35}S-labeled proteoglycans to digestion by *Flavobacterium heparinum* heparinase (Miles Laboratories) was determined in one experiment according to the procedure of Linker and Hovingh (21). Heparinase (~5 units) was added to 275 μl of 1 mM calcium acetate and 100 mM sodium acetate, pH 7.0, containing ^{35}S-labeled proteoglycans (5000 cpm), 100 μg each of heparin and chondroitin sulfate A glycosaminoglycan carriers, and 1% (v/v) of the antibacterial agent toluene. Following incubation of the samples at 37 °C for 16 h, the extent of digestion by heparinase was assessed by PD-10 analysis.

^{35}S-Labeled chondroitin sulfate glycosaminoglycans were detected by their susceptibility to digestion with chondroitinase ABC according to the procedure of Saito *et al.* (22). Samples of ^{35}S-labeled proteoglycans (800–3000 cpm) were incubated in a 300-μl final volume at 37 °C for 1 h with 0.4 units of *Proteus vulgaris* chondroitinase ABC (Miles Laboratories) in the presence of 50 mM Tris-HCl, 35 mM sodium acetate, 50 mM NaCl, 100 mg/ml bovine serum albumin, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 0.06 μg/ml pepstatin, 1 mM *N*-ethylmaleamide, and 100 μg chondroitin sulfate A carrier, pH 8.0. The reaction was terminated by the addition of an equal volume of 8 M guanidine-HCl, and the percentage of ^{35}S-labeled proteoglycans digested to unsaturated ^{35}S-labeled disaccharides was assessed by PD-10 chromatography. Alternatively, chondroitinase ABC digestion was stopped by the addition of 4 volumes of ethanol. These samples were placed on ice for 20 min, centrifuged at 8000 × *g* for 5 min, and the supernatants were concentrated under nitrogen; the ^{35}S-labeled unsaturated disaccharides were identified by the high-performance liquid chromatography (HPLC) method of Seldin *et al.* (23).

Gel Filtration Chromatography and Isoelectric Focusing of [^{3}H] DFP-binding Proteins Exocytosed from Activated BMMC—To determine the hydrodynamic size of the exocytosed [^{3}H]DFP-binding proteins, 1–3 ml of supernatants from $1–3 \times 10^7$ calcium ionophore-activated BMMC were resolved by Sepharose CL-2B gel filtration as described for the proteoglycans. Each gel filtration fraction was incubated at 37 °C for 1 h with 1 μCi [1,3-^{3}H]DFP (4 Ci/mmol, Amersham Corp.), and the resulting [^{3}H]DFP-labeled proteins were separated from unbound radioactivity by PD-10 gel filtration chromatography as described above. The radioactivity filtering in the void volume of the PD-10 gel filtration column was considered to be due to the presence of [^{3}H]DFP-labeled proteins (14). Alternatively, [^{3}H] DFP-labeled proteins were concentrated by trichloroacetic acid precipitation and their M_r determined by SDS-PAGE using the technique of Laemmli (24) as modified by DuBuske *et al.* (14). Following electrophoresis, gels were cut vertically into lanes. The lanes were sectioned horizontally at 2.5-mm intervals, and each section was dissolved by heating at 65 °C for 3 h in 1.5 ml of 28% (v/v) H_2O_2 containing 1.12% NH_4OH. The radioactivity was determined by β-scintillation counting after the addition of 10 ml of Hydrofluor.

To determine the effect of removal of the proteoglycans on hydrodynamic size of the [^{3}H]DFP-binding proteins, supernatants from calcium ionophore-activated BMMC were made 5 M in NaCl and were applied to a 10 × 10-mm phenyl-Sepharose CL-4B column (Pharmacia) that was equilibrated in 5 M NaCl, 0.01 M Tris-HCl, pH 7.2. The hydrophobic column was washed with 15 ml of the high salt equilibration buffer to ensure complete removal of the proteoglycans; the retained proteins were eluted with 3 ml of 0.01 M Tris-HCl, pH 7.2. The eluates containing the [^{3}H]DFP-binding proteins were applied to the Sepharose CL-2B gel filtration column under the nondissociating conditions as described previously, and the filtration was followed by subsequent [^{3}H]DFP-labeling of the column fractions. Because of the poor recovery on Sepharose CL-2B of the [^{3}H]DFP-labeled proteins when separated from the proteoglycans, 20 μg/ml of cytochrome *c* (horse heart type III, Sigma) was added to the elution buffer. For comparison, the 30,000 M_r marker [^{14}C]carbonic anhydrase (New England Nuclear) was separately applied to the Sepharose CL-2B column; its filtration from the Sepharose CL-2B column was determined by β-scintillation counting of the column fractions.

For isoelectric focusing, supernatant from activated BMMC was labeled with 10 μCi/ml of [^{3}H]DFP at 37 °C for 1 h; the [^{3}H]DFP-labeled proteins were separated from the unbound [^{3}H]DFP and the proteoglycans by hydrophobic interaction chromatography on phenyl-Sepharose as described above. Following dialysis against water, the eluted [^{3}H]DFP-labeled proteins were lyophilized and resuspended in

50 μl of 2.4% (w/v) pH 3–10 ampholytes (FMC, Rockland, ME), 8 M urea, 0.3 M sucrose, 10% (v/v) Nonidet P-40, and 0.01% (w/v) bromphenol blue. Isoelectric focusing was performed by a modification of the technique of Ferreira and Eichinger (25) in order to achieve a higher pH range (26). After application of sample, the gel was subjected to 10 mA constant current until 800 V was achieved, and this voltage was maintained for 4 h. Following isoelectric focusing, the gel was cut vertically into 1-cm lanes. To determine the pH gradient, one lane was sectioned horizontally at 1-cm intervals; each section was placed in 1 ml of distilled water and allowed to equilibrate for 30 min before the pH was measured. To detect the location of the [^{3}H]DFP-labeled proteins, the sample lane was sectioned horizontally at 2.5-mm intervals; the gel sections were dissolved by H_2O_2/NH_4OH treatment, and their radioactivity was determined.

Samples containing [^{3}H]DFP-binding proteins were assessed for proteolytic activity by incubation with human fibronectin for 0–4 h at a substrate/enzyme ratio of at least 200:1; the protein concentration of the latter was estimated by [^{3}H]DFP-binding capacity, as described (14). Degradation of fibronectin was quantified by loss of the 440,000 M_r molecule as assessed by SDS-PAGE (14).

RESULTS

Hydrodynamic Size and Glycosaminoglycan Composition of the Proteoglycans Exocytosed from BMMC—In nine experiments utilizing calcium ionophore-activated, ^{35}S-labeled BMMC, the release of ^{35}S-labeled proteoglycans and β-hexosaminidase was 45 ± 13% (mean ± SD) and 57 ± 13%, respectively, whereas the release of the cytosolic marker lactate dehydrogenase was 5.2 ± 3.0%. The ^{35}S-labeled proteoglycans present in the supernatants of the activated BMMC filtered on a Sepharose CL-2B column equilibrated in physiologic salt as one minor and one major peak (Fig. 1*A*). In six experiments, 5.4 ± 1.4% (mean ± SD) of the total ^{35}S-labeled macromolecules were recovered in the excluded volume of the column ($M_r > 1 \times 10^7$), and the remainder of the ^{35}S-labeled proteoglycans eluted in the included volume with a K_{av} of 0.66, indicative of ~200,000 M_r. Two approaches were used to establish that the filtration profile of the newly synthesized ^{35}S-labeled proteoglycans was representative of the nonradiolabeled proteoglycans that also were exocytosed. In order to determine if the percentage of the ^{35}S-labeled proteoglycan in the excluded volume changed with time, the BMMC were incubated with [^{35}S]sulfate, washed, chased for 24 h in radioisotope-free medium, and then activated. 7% of the total released ^{35}S-labeled proteoglycans resolved by Sepharose CL-2B filtration were recovered in the excluded volume. In a separate experiment, the amount of proteoglycan in each Sepharose CL-2B fraction was determined by colorimetric assay; ~12% of the exocytosed uronic acid-positive molecules from the activated BMMC were recovered in the excluded volume of the Sepharose CL-2B column.

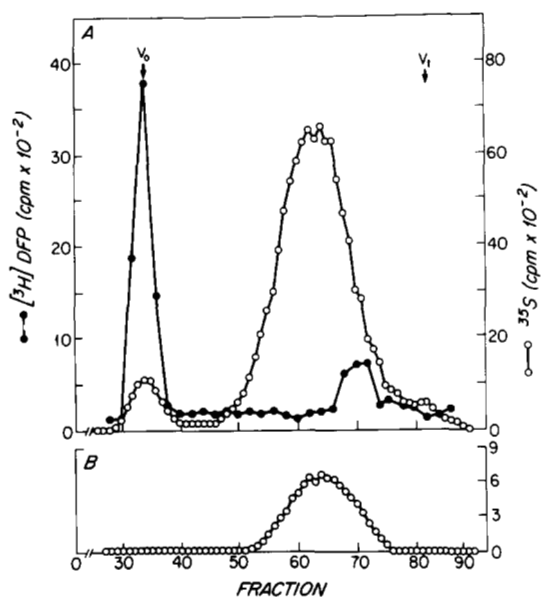

FIG. 1. ***A*, Sepharose CL-2B chromatography under nondissociative conditions of exocytosed ^{35}S-labeled proteoglycans (○——○) and [^{3}H]DFP-labeled proteins (●——●) present in the supernatants of calcium ionophore A23187-activated mouse BMMC; *B*, Sepharose CL-2B chromatography of the ^{35}S-labeled proteoglycans (○——○) in the large M_r complex after removal of the proteases by hydrophobic chromatography.** In these representative experiments, the recoveries of [^{3}H]DFP-labeled proteins and ^{35}S-labeled proteoglycans were 96 and 98%, respectively. The void (V_o) and total volumes (V_t) of the column are indicated.

To dissociate the large M_r complex, pooled fractions from the excluded volume of the Sepharose CL-2B column were made 5 M in NaCl and applied to a phenyl-Sepharose CL-4B column. The ^{35}S-labeled proteoglycans appeared in the 5 M NaCl effluent. After dialysis, lyophilization, and suspension in the 0.15 M NaCl buffer, these proteoglycans filtered in the included volume of the Sepharose CL-2B column as a single peak with a K_{av} of 0.66 (Fig. 1*B*). This K_{av} is identical to that of the proteoglycans that originally filtered in the included volume of the column.

The ^{35}S-labeled proteoglycans present in the excluded and included volumes of the Sepharose CL-2B column were each subjected to separate digestion with chondroitinase ABC, nitrous acid, and heparinase, and the extent of degradation was quantified by PD-10 chromatography. In three experiments, the ^{35}S-labeled proteoglycans excluded from the Sepharose CL-2B column contained approximately equal amounts of chondroitin sulfate glycosaminoglycans and heparin-like glycosaminoglycans, whereas the included proteoglycans contained almost exclusively chondroitin sulfate glycosaminoglycans (Table I). Following chondroitinase ABC digestion and HPLC analysis, 38 and 62% of the ^{35}S-labeled unsaturated disaccharides derived from the excluded proteoglycans, and 41 and 53% of the ^{35}S-labeled unsaturated disaccharides from the included proteoglycans depicted in Fig. 1, eluted at the retention times that corresponded to ΔDi-4S (2-acetamido-2-deoxy-3-*O*-[β-D-gluco-4-enepyranosyluronic acid]-4-*O*-sulfo-D-galactose) and ΔDi-diS_E (2-acetamido-2-deoxy-3-*O*-[β-D-gluco-4-enepyranosyluronic acid]-4-6-di-*O*-sulfo-D-galactose) respectively (Fig. 2).

To determine whether or not the chondroitin sulfate E and heparin glycosaminoglycans were attached to distinct peptide cores, the ^{35}S-labeled proteoglycans derived from the excluded volume of the Sepharose CL-2B column were subjected to Sepharose CL-4B gel filtration chromatography under the dissociative conditions of 4 M guanidine-HCl before and after treatment with chondroitinase ABC. Chondroitinase ABC

TABLE I

Susceptibility to degradation of the ^{35}S-labeled proteoglycans exocytosed from calcium ionophore-activated BMMC

^{35}S-proteoglycan[a]	Degradation method			
	None	Chondroitinase	Nitrous acid	Heparinase
Excluded	0.4 ± 0.5	54 ± 20	42 ± 18	48
Included	0.4 ± 0.5	91 ± 2	7 ± 2	1

[a] The exocytosed proteoglycans were resolved into two subpopulations by Sepharose CL-2B gel filtration, one that filtered in the excluded volume and one that filtered in the included volume of the column, as depicted in Fig. 1. The values are expressed as the percentage of the ^{35}S-labeled proteoglycans degraded by the indicated digestion method. Each value is the mean ± S.D. with $n = 3$, except for heparinase treatment in which $n = 1$.

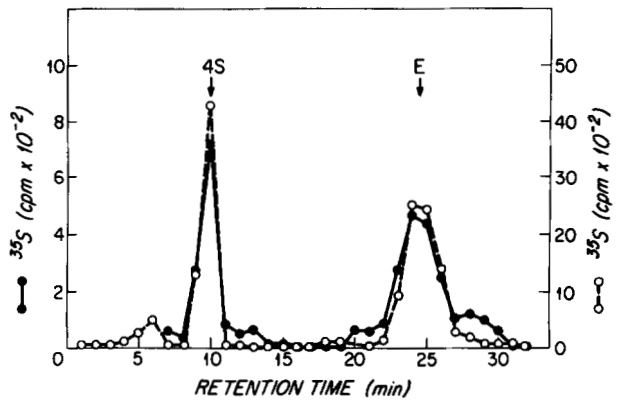

FIG. 2. **HPLC analyses of the ^{35}S-labeled disaccharides of exocytosed proteoglycans.** Unsaturated ^{35}S-labeled disaccharides were generated by chondroitinase ABC treatment of the released ^{35}S-labeled proteoglycans that filtered in the void volume (●——●) and the included volume (O---O) of the Sepharose CL-2B column (Fig. 1) and then were analyzed by HPLC. The retention times of the standard disaccharides, ΔDi-4S (4S) and ΔDi-diS_E (E), are indicated.

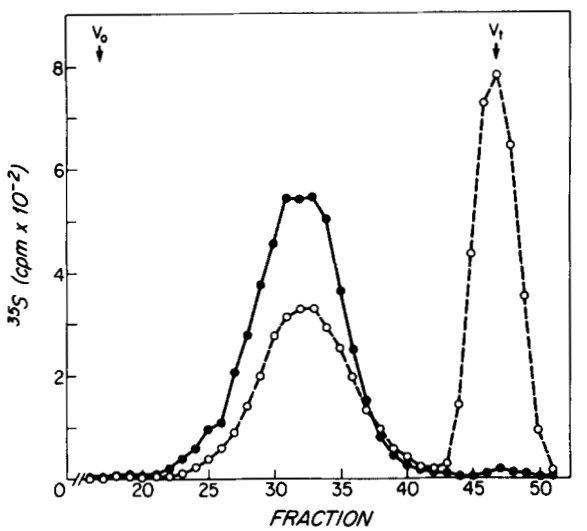

FIG. 3. **Sepharose CL-4B chromatography under dissociative conditions of exocytosed ^{35}S-labeled proteoglycans in the high M_r complex.** The ^{35}S-labeled proteoglycans excluded by Sepharose CL-2B filtration were chromatographed on Sepharose CL-4B before (●——●) and after (O---O) incubation with chondroitinase ABC. The void (V_0) and total volumes (V_t) are indicated.

treatment resulted in removal of the chondroitin sulfate E side chains, as indicated by the shift of 48% of the radioactivity to the elution position of ^{35}S-labeled disaccharides. This did not alter the K_{av} of the ^{35}S-labeled proteoglycans that contained heparin-like side chains (Fig. 3) and indicates that the heparin proteoglycans possess a M_r of ~200,000.

Characterization by Size and Isoelectric Focusing of [^{3}H] DFP-Binding Proteins Exocytosed from Activated BMMC— In three experiments, the percent release of [^{3}H]DFP-binding proteins and β-hexosaminidase was 34 ± 5.3% (mean ± SD) and 64 ± 6.7%, respectively; the release of lactate dehydrogenase was 3.0 ± 0.5%. In the representative experiment depicted in Figure 1*A*, 78% of the total [^{3}H]DFP-labeled proteins present in the supernatants of calcium ionophore-activated BMMC filtered in the excluded volume of the Sepharose CL-2B column. In three experiments, 71 ± 6% (mean ± SD) of the exocytosed [^{3}H]DFP-binding proteins were recovered in the excluded volume, indicating filtration as a complex with >1 × 10^7 M_r. Upon analysis by SDS-PAGE, the [^{3}H]DFP-binding proteins present in both the excluded and included volumes electrophoresed with ~30,000 M_r. Following removal of the proteoglycans by chromatography on phenyl-Sepharose CL-4B, the [^{3}H]DFP-binding proteins filtered as a single peak at V_t when applied to the Sepharose CL-2B gel filtration column under nondissociating conditions, whereas the 30,000 M_r marker, [^{14}C]carbonic anhydrase, filtered slightly ahead of V_t with a K_{av} of 0.89. When the [^{3}H]DFP-labeled exocytosed proteins were separated from the proteoglycans and subjected to isoelectric focusing, a predominant peak of [^{3}H]DFP-labeled protein was observed with a pI ~9.1 (Fig. 4).

Supernatant from ionophore-activated BMMC and the fraction in the excluded volume from the Sepharose CL-2B column were each incubated with fibronectin and the resulting digestion products were analyzed by SDS-PAGE. For both samples, ~50% of the 440,000 M_r native fibronectin was degraded within 2 h into cleavage products of 210,000, 200,000, 190,000, 145,000, 135,000, 100,000, and 27,000 M_r; the cleavage products were identical for both of the samples and similar to those previously reported for the intragranular serine proteases of the mouse BMMC (14).

DISCUSSION

The current study demonstrates that calcium ionophore-activated mouse BMMC exocytose secretory granule [^{3}H] DFP-binding proteins of which 71 ± 6% (mean ± SD, n = 3) are bound to heparin and chondroitin sulfate proteoglycans in complexes with M_r > 1 × 10^7 (Fig. 1*A*) as determined by Sepharose CL-2B gel filtration under nondissociating conditions. Upon removal of the proteoglycans and rechromatography on Sepharose CL-2B, the [^{3}H]DFP-binding proteins filtered with a M_r no greater than 30,000, indicating that the [^{3}H]DFP-binding proteins are not aggregated to one another as multimers. By SDS-PAGE analysis, the [^{3}H]DFP-binding proteins that were associated with the large M_r complex were ~30,000 M_r, which is the same value as previously reported for mouse BMMC intragranular serine proteases (14). Because the predominant intragranular [^{3}H]DFP-binding proteins were demonstrated to be cationic with a pI ~9.1 (Fig. 4), it is likely that ionic interactions provide the basis for formation of the macromolecular complex. When the high M_r complexes were not resolved prior to isoelectric focusing, the [^{3}H]DFP-labeled proteins did not enter the polyacrylamide

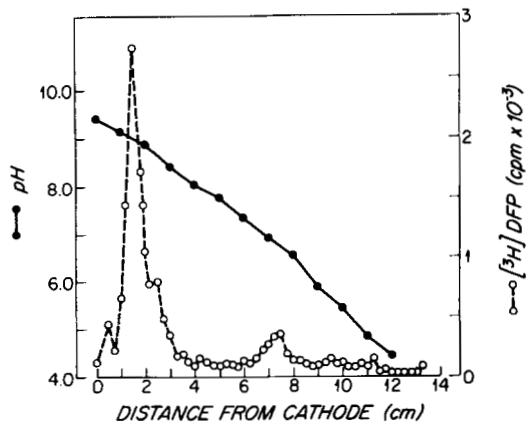

FIG. 4. **Isoelectric focusing of [^{3}H]DFP-binding proteins exocytosed from calcium ionophore A23187-activated mouse BMMC.** The pH (●——●) of each fraction was determined at 1-cm intervals. [^{3}H]DFP-labeled proteins were detected by β-scintillation counting of 2.5-mm sections of the gel (O---O).

gel because of their large apparent hydrodynamic size (data not shown).

As assessed by Sepharose CL-2B chromatography under physiologic salt conditions, 5.4 ± 1.4% (mean ± SD, $n = 6$) of the exocytosed ^{35}S-labeled proteoglycans filtered in the excluded volume of this column (Fig. 1*A*). That the filtration profile of the newly synthesized ^{35}S-labeled proteoglycans was representative of stored, nonlabeled proteoglycans was confirmed by a pulse-chase experiment and by measurement of the uronic acid content of the Sepharose CL-2B column fractions. When the ^{35}S-labeled proteoglycans in the large M_r complex were separated from the [^{3}H]DFP-binding proteins by high salt treatment and hydrophobic chromatography and reapplied to the Sepharose CL-2B gel filtration column, the ^{35}S-labeled proteoglycans filtered in the included volume with a $K_{av} = 0.66$, indicating an M_r of ~200,000 (Fig. 1*B*). This hydrodynamic size is identical to that of the predominant secreted ^{35}S-labeled proteoglycans (Fig. 1*A*) and is similar to that of purified mouse BMMC chondroitin sulfate E proteoglycan (7). Furthermore, upon disaccharide analysis both the excluded and the included chondroitin sulfate proteoglycans contained about 43% Di-diS_E (glucuronic acid → *N*-acetylgalactosamine-4,6-disulfate) when corrected for two sulfate residues per disaccharide (Fig. 2), which is indicative of chondroitin sulfate E (6).

However, in addition to chondroitin sulfate E, 42 ± 18% (mean ± SD, $n = 3$) of the proteoglycans present in the high M_r complex contained nitrous acid/heparinase-susceptible heparin glycosaminoglycans (Table I). That the heparin and chondroitin sulfate E glycosaminoglycan side chains were attached to different peptide cores was established by Sepharose CL-4B gel filtration chromatography performed under dissociative conditions before and after removal of the chondroitin sulfate side chains by chondroitinase ABC. Untreated proteoglycans filtered as a single symmetrical peak with a 0.48 K_{av}. After removal of the chondroitin sulfate side chains, the remaining heparin-containing proteoglycans again filtered with a 0.48 K_{av} (Fig. 3). Thus, rather than containing a hybrid molecule such as that in the secretory granules of the RBL-1 cells (27), the granules of the mouse BMMC contain two species of proteoglycans of 200,000 M_r, one that contains predominantly chondroitin sulfate E glycosaminoglycans and one that contains heparin glycosaminoglycans. In previous analyses of mouse BMMC proteoglycans (6), the heparin proteoglycan was not appreciated because it represents only ~3% of the total intragranular proteoglycan. However, the preferred association of heparin proteoglycan with the high M_r complex allowed its isolation and ready identification in the current study. The preferential binding of heparin proteoglycan to the cationic proteins indicates either that this complex originates from a subpopulation of heparin-containing mast cells in the culture, or that the proteins are packaged in the granules of cells with both types of proteoglycans, and the selective association occurs because heparin has a higher affinity for the [^{3}H]DFP-binding proteins than chondroitin sulfate E. It was earlier observed that rat serosal mast cells which actively synthesize only heparin proteoglycans *ex vivo* possess the biosynthetic machinery to polymerize chondroitin sulfate E onto the glycosaminoglycan acceptor, *p*-nitrophenyl-β-D-xyloside (28). The recognition of both glycosaminoglycan enzymatic pathways in two distinct mast cell subclasses suggests that proteoglycan biosynthesis may be regulated by the expression of one of two different peptide cores or by post-translational modification in the Golgi of a common peptide core.

Upon activation of rat serosal mast cells, heparin proteoglycan is exocytosed as a complex that is bound to both carboxypeptidase A (29) and the serine protease, chymase (11, 12). The exocytosis of mast cell proteases in large M_r complexes bound to secretory granule proteoglycans may prevent extensive diffusion of the enzymes, impair their inactivation by protein inhibitors, or both. Since heparin proteoglycan-protease complexes released from rat serosal mast cells are avidly phagocytosed by macrophages (30), eosinophils (31), and fibroblasts (32), it is also possible that these large M_r complexes have evolved to target delivery of the mast cell serine proteases to adjacent cells without indiscriminate degradation of connective tissue.

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