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Secretory Granules of Heparin-containing Rat Serosal Mast Cells also Possess Highly Sulfated Chondroitin Sulfate Proteoglycans*

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Rat serosal mast cells, which synthesize only heparin proteoglycans as detected by intrinsic labeling with [^{35}S]sulfate, were analyzed for the presence of intracellular chondroitin sulfate proteoglycans by chemical and immunochemical means. Rat serosal mast cells of >99% purity were treated with Zwittergent 3-12 and 4 M guanidine HCl, and the extracted nonradiolabeled proteoglycans were purified by density gradient centrifugation. As assessed by quantification of the unsaturated disaccharides released from the proteoglycans by chondroitinase ABC treatment, 10^6 rat serosal mast cells contained 2.4–4.5 μg of chondroitin sulfate proteoglycans. Analysis of the chondroitinase ABC digests by high performance liquid chromatography revealed the unsaturated disaccharides ΔDi-4S, ΔDi-diS$_B$, and ΔDi-diS$_E$ which were derived from GlcA→GalNAc-4-SO$_4$, iduronic acid-2-SO$_4$→GalNAc-4-SO$_4$, and GlcA→GalNAc-4,6-diSO$_4$, respectively. The molar ratio of the monosulfated to disulfated disaccharides was ~2:1 with ΔDi-diS$_E$ > ΔDi-diS$_B$. When analyzed with a mouse anti-chondroitin sulfate monoclonal antibody and fluorescein-labeled F(ab')$_2$ goat anti-mouse IgG, ~91% of permeabilized and chondroitinase ABC-treated cells in the mast cell preparations exhibited intracellular fluorescence, and the pattern of staining indicated that the chondroitin sulfate molecules were located in the secretory granules. The specificity of the monoclonal antibody for the unsaturated double bond created by chondroitinase ABC treatment of the proteoglycan *in situ* was established by the absence of fluorescence when the chondroitinase ABC step was omitted or when heparinase digestion was substituted for chondroitinase ABC. Furthermore, the ability of the anti-chondroitin sulfate monoclonal antibody to mediate fluorescence *in situ* was markedly reduced by absorption with solid-phase chondroitin sulfate proteoglycan that had been chondroitinase ABC-treated, but not by absorption with undigested proteoglycan or with solid-phase heparin. The highly sulfated chondroitin sulfate proteoglycans of rat serosal mast cells are the same type synthesized by the rat mucosal mast cell subclass. The simultaneous presence of both heparin and chondroitin sulfate glycosaminoglycans in rat serosal mast cells in the absence of detectable chondroitin sulfate synthesis suggests that this chondroitin sulfate-containing proteoglycan is a remnant of an earlier stage of differentiation and implies an ontologic relationship between the two mast cell subclasses.

Distinct subpopulations of mast cells in the rat have been identified by the differences in the types of proteoglycans (1, 2) and serine proteases (3, 4) that they synthesize and store in their secretory granules, by their differential histochemical staining properties (5), by their relative histamine content per cell (6, 7), by the presence or absence of T-cell dependence for maintenance (8), and by the relative ability of various agonists to elicit release of preformed intragranular mediators (7). Mast cells obtained from the serosal surfaces of the rat peritoneal cavity incorporate [^{35}S]sulfate into a protease-resistant M_r 750,000 heparin proteoglycan (1), whereas T-cell-dependent mast cells isolated from the mucosa of the small intestine of *Nippostrongylus brasiliensis*-infected rats incorporate [^{35}S]sulfate into a protease-resistant M_r 150,000 chondroitin sulfate proteoglycan (2). When the chondroitin sulfate proteoglycans from rat mucosal mast cells are incubated with chondroitinase ABC and the generated unsaturated disaccharides are analyzed by high performance liquid chromatography (HPLC[1]), the three disaccharides ΔDi-4S, ΔDi-diS$_B$, and ΔDi-diS$_E$ are detected with the concentration of ΔDi-4S = ΔDi-diS$_B$ > ΔDi-diS$_E$. Although rat serosal mast cells do not synthesize detectable levels of ^{35}S-labeled chondroitin sulfate proteoglycans *ex vivo*, they synthesize chondroitin sulfate glycosaminoglycans that are rich in Di-diS$_E$ onto the exogenous glycosaminoglycan acceptor, *p*-nitrophenyl-β-D-xyloside, during short-term culture (9).

We now demonstrate by chemical techniques that rat serosal mast cells have chondroitin sulfate proteoglycans and that their chondroitin sulfate glycosaminoglycans contain the same types of unique disulfated disaccharides that are detected by ^{35}S-labeling of rat mucosal mast cells. Furthermore, using a novel monoclonal anti-chondroitin sulfate antibody for intracellular immunofluorescence staining, we have determined that the chondroitin sulfate molecules are in essentially all rat serosal mast cells and are located intragranularly.

EXPERIMENTAL PROCEDURES

Chemical Characterization of Chondroitin Sulfate in Rat Serosal Mast Cells—Rat serosal mast cells were obtained by lavage of the

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; ΔDi-4S, ΔDi-6S, ΔDi-diS$_B$, ΔDi-diS$_D$, and ΔDi-diS$_E$, the unsaturated disaccharides derived from GlcA→GalNAc-4-SO$_4$, GlcA→GalNAc-6-SO$_4$, iduronic acid-2-SO$_4$→GalNAc-4-SO$_4$, GlcA-2-SO$_4$→GalNAc-6-SO$_4$, and GlcA→GalNAc-4,6-diSO$_4$, respectively; HBSS, Hanks' balanced salt solution.

peritoneal cavity of each rat with 30 ml of Tyrode's buffer containing 1 mg/ml gelatin. The mast cells were concentrated to >99% purity by one isopyknic (1) and two isokinetic (10) centrifugations. Each preparation of 1.1–1.3 × 10^8 highly purified mast cells from 100 rats was suspended in 150 μl of 1% (w/v) Zwittergent 3-12 (Behring Diagnostics) for ~15 s and then in 12 ml of 4 M guanidine HCl containing 0.01 M Tris-HCl and CsCl, pH 7.0 (final density = 1.4 g/ml) (11). The mast cell extracts were centrifuged for 48 h at ~100,000 × *g*, and the bottom halves of the gradients were dialyzed sequentially against 1 M sodium acetate followed by 0.1 M ammonium bicarbonate; the dialyzed samples were lyophilized and resuspended in 2 ml of H_2O. The total amount of proteoglycan in each preparation was quantified by determining the uronic acid content of 5–25 μl of the density gradient-purified samples by the carbazole technique (12). To determine the amount of chondroitin sulfate, 100–150-μl samples were suspended in 5 mM Tris-HCl, 5 mM sodium acetate, 5 mM NaCl, and 0.01% bovine serum albumin (enriched-Tris buffer), and were incubated with or without 0.05 unit of chondroitinase ABC (Miles Laboratories) for 30 min at 37 °C. The chondroitinase ABC digests were analyzed for changes in their absorbance at 232 nm, and the amount of chondroitin sulfate was calculated by the method of Saito *et al.* (13) using an extinction coefficient of 5.1/μmol for the generated unsaturated disaccharides. Replicate chondroitinase ABC-treated samples were each made 80% in ethanol, placed in an ice bath for 30 min, and centrifuged at 8,000 × *g* for 5 min at 4 °C. The unsaturated disaccharides in the ethanol extract were then resolved by HPLC and quantified by monitoring the eluate at 232 nm (14). The unsaturated disaccharides derived from GlcA-2-SO_4→GalNAc-6-SO_4 (ΔDi-diS_D) and ΔDi-diS_E were prepared from shark cartilage and squid cartilage chondroitin sulfates, respectively, and were used as standards. In some preparations, the amount of chondroitin sulfate in the samples was also estimated by its integrated absorbance relative to that of known amounts of ΔDi-6S, the unsaturated disaccharide derived from GlcA→GalNAc-6-SO_4 (Miles), which was coinjected with the sample.

Immunologic Demonstration of Chondroitin Sulfate in Isolated Rat Serosal Mast Cells—An intracellular immunofluorescent technique employing monoclonal antibodies was used to determine the percentage of the cells in the rat serosal mast cell population that contained intragranular chondroitin sulfate proteoglycans. The monoclonal antibody used (5/29/2-B-6) was of the IgG_1 subclass and was obtained by immunizing mice with chondroitinase ABC-treated bovine nasal cartilage proteoglycan (15). This high affinity antibody has analogous specificity with two other monoclonal antibodies (4/8/9-A-2 and 4/8/2-B-4) that specifically recognize oligosaccharides containing nonreducing terminal disaccharides of ΔDi-4S (15, 16). Purified rat serosal mast cells were suspended in Hanks' balanced salt solution (HBSS) and were permeabilized by the addition of absolute ethanol to a final concentration of 90% (v/v). The permeabilized mast cells were spread on cytocentrifuge slides and treated with HBSS, 0.25 unit of chondroitinase ABC in HBSS, or 25 units of heparinase (Miles) in HBSS for 15 min at 37 °C. Following two washes with HBSS containing 0.1% bovine serum albumin and 0.02% sodium azide (H/B/A), the mast cells were incubated for 15 min at 25 °C with a saturating dilution (6.6 μg/ml mouse IgG_1) of either the anti-chondroitin sulfate monoclonal antibody or an equal dilution of an anti-keratan sulfate monoclonal antibody, 1/20/5-D-4 (16). The cell preparations were washed twice with H/B/A, and incubated for 5 min at 25 °C with a 1:80 dilution of fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (Cooper Biomedical, Malvern, PA). Following two additional washes with H/B/A, a drop of phosphate-buffered saline/glycerol (1:1, v/v) was placed over the cells, and the preparations were covered with coverslips. The mast cells were photographed with a Leitz Orthoplan epi-illumination fluorescence microscope also equipped for Nomarski interference contrast microscopy. The anti-keratan sulfate monoclonal antibody was chosen as a negative control reagent because rat serosal mast cells do not contain detectable amounts of this glycosaminoglycan and because this antibody is of the same subclass as the anti-chondroitin sulfate antibody.

Heparin-agarose (Sigma) and chondroitin sulfate proteoglycan-Sepharose 4B were used as affinity resins to ensure the specificity of the 5/29/2-B-6 monoclonal antibody. Sixteen mg of rat chondrosarcoma chondroitin sulfate proteoglycans were dissolved in 20 ml of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3) and incubated overnight at 4 °C with 1 g of cyanogen bromide-activated Sepharose 4B (Sigma). The gel was separated from the supernatant by centrifugation. As assessed by a decrease in the uronic acid content in the supernatant, it was estimated that ~25% of the added chondrosarcoma chondroitin sulfate proteoglycans were coupled to the resin. The coupled gel was incubated for 2 h at 25 °C with 1 M ethanolamine, pH 8.0, and then was washed five times on a sintered glass funnel alternately with coupling buffer and 0.5 M NaCl, 0.1 M sodium acetate, pH 4.0. Two ml of chondroitin sulfate proteoglycan-Sepharose affinity resin were suspended in enriched-Tris buffer with protease inhibitors (17) and incubated with 1 unit of chondroitinase ABC for 30 min at 37 °C; the digestion was stopped by washing the gel with HBSS. Samples (100 μl) of the 5/29/2-B-6 antibody (6.6 μg of IgG_1/ml) were mixed for 1 h at room temperature with 25 μl of packed chondroitin sulfate proteoglycan-Sepharose (containing an estimated 12 μg of chondroitin sulfate glycosaminoglycans), 25 μl of packed chondroitin sulfate proteoglycan-Sepharose that had been treated with chondroitinase ABC, or 15.7 μl of packed heparin-agarose (containing an estimated 12 μg of heparin glycosaminoglycans). The supernatants were recovered by centrifugation and the capacities of serial dilutions of these supernatants to exhibit intracellular immunofluorescent staining of permeabilized, chondroitinase ABC-treated rat serosal mast cells were compared to each other and to similar dilutions of unabsorbed antibody.

RESULTS

Chemical Characterization of Chondroitin Sulfate in Rat Serosal Mast Cells—As assessed by the carbazole reaction, the density gradient-purified extracts contained ~25 μg of uronic acid per 10^6 rat serosal mast cells. Incubating 200 μg of this material with chondroitinase ABC resulted in a 0.405 net change in absorbance at 232 nm. Based on the reported extinction coefficient of ΔDi-4S, samples from two separate experiments contained ~43 and ~27 μg of chondroitin sulfate, thereby allowing the estimate that 10^6 rat serosal mast cells contained 4.5 ± 1.0 μg (mean ± range, n = 2) of chondroitin sulfate proteoglycans.

Resolution by HPLC of the unsaturated disaccharides in the chondroitinase ABC digests of density gradient-purified proteoglycans revealed peaks of absorbance at retention times of 8.9, 12.8, and 13.8 min (Fig. 1), corresponding to the respective standards ΔDi-4S, ΔDi-diS_B, and ΔDi-diS_E. As the resolution between the ΔDi-diS_B and ΔDi-diS_E unsaturated disaccharides was not complete, the ratio of ΔDi-4S to the sum of the disulfated disaccharides was analyzed in three separate experiments, with the results being 2.3:1.0 (Fig. 1), 1.4:1.0, and 2.0:1.0. In each case, the findings were comparable to Fig. 1 in that the quantity of ΔDi-diS_E was somewhat greater than ΔDi-diS_B. By comparing the relative areas of the ΔDi-6S and ΔDi-4S peaks when known amounts of ΔDi-6S were co-injected with another aliquot of this sample, it was estimated that 10^6 rat serosal mast cells contained 2.4 ± 0.4

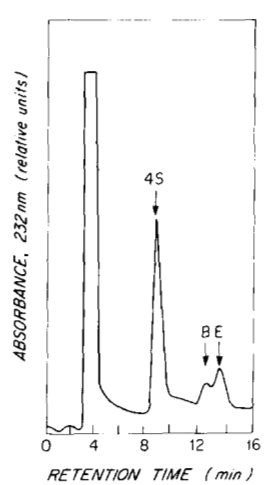

FIG. 1. **HPLC of a chondroitinase ABC digest of density gradient-purified proteoglycans from rat serosal mast cells.** The retention times of the standard disaccharides ΔDi-4S (*4S*), ΔDi-diS_B (*B*), and ΔDi-diS_E (*E*) are indicated.

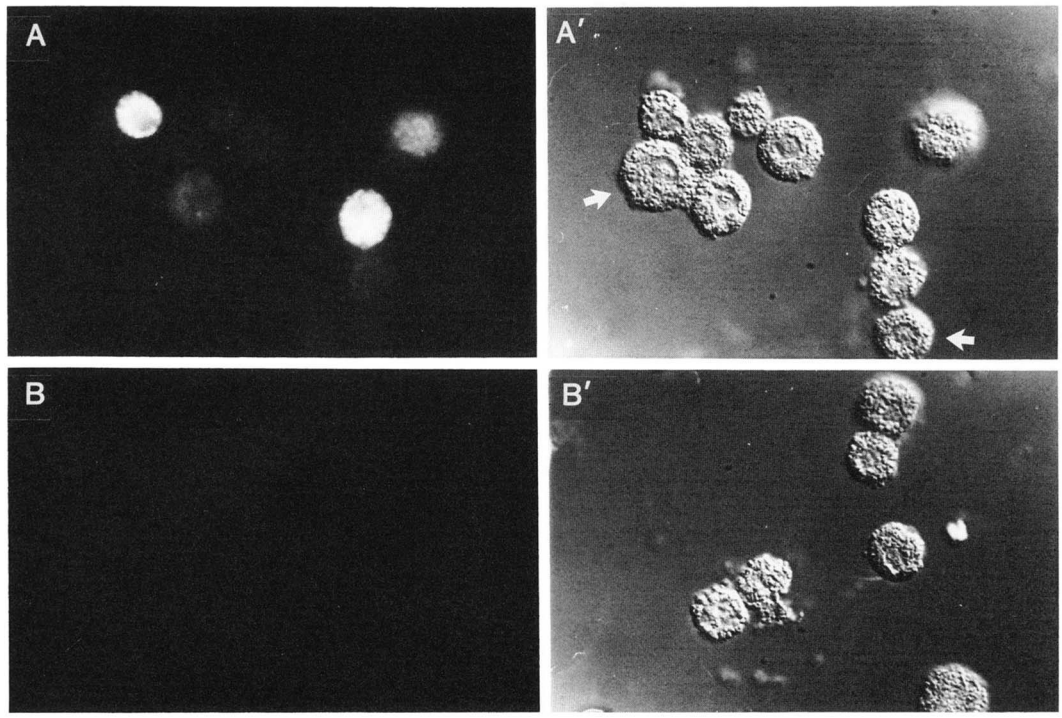

FIG. 2. **Fluorescence (*A*, *B*) and paired Nomarski interference microscopy (*A'*, *B'*) photomicrographs of permeabilized rat serosal mast cells stained with anti-glycosaminoglycan monoclonal antibodies.** Cells were treated with chondroitinase ABC and then incubated with either anti-chondroitin sulfate (*A*) or anti-keratan sulfate (*B*) monoclonal antibodies before being stained with fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG. The *arrows* in *panel A'* identify two cells that failed to fluoresce and are not seen in *panel A*. Magnification, × 1043.

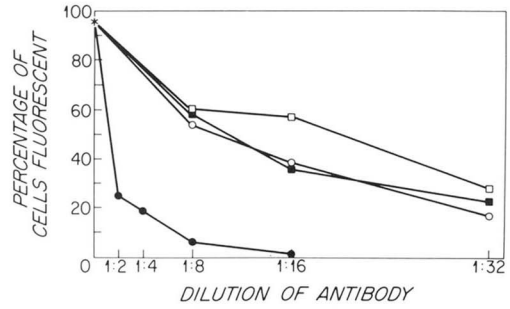

FIG. 3. **Immunofluorescence of permeabilized, chondroitinase ABC-treated rat serosal mast cells stained with dilutions of unabsorbed anti-chondroitin sulfate 5/29/2-B-6 monoclonal antibody (□), 5/29/2-B-6 after absorption with heparin-agarose (■), or 5/29/2-B-6 after absorption with nontreated (○) or chondroitinase ABC-treated (●) rat chondrosarcoma chondroitin sulfate proteoglycan coupled to Sepharose 4B.** The binding of undiluted, unabsorbed antibody is indicated by the *asterisk*. Data are from one representative experiment.

μg (mean ± range, $n = 2$) of chondroitin sulfate.

Intracellular Detection by Immunofluorescence Microscopy of Chondroitin Sulfate in Rat Serosal Mast Cells—Incubating permeabilized, chondroitinase ABC-treated rat serosal mast cells with a saturating amount of the anti-chondroitin sulfate 5/29/2-B-6 monoclonal antibody and then with fluoresceinated $F(ab')_2$ goat anti-mouse IgG resulted in the staining of 91 ± 4.5% (mean ± S.D., $n = 3$) of the cells; the intensity of staining ranged from dim to extremely bright (Fig. 2*A*). The fluorescence was intracellular and granular in all positive cells. The specificity of the 5/29/2-B-6 monoclonal antibody for the unsaturated double bond created in the chondroitin sulfate glycosaminoglycan after treatment with chondroitinase ABC was confirmed *in situ* by the finding that permeabilized cells not treated with chondroitinase ABC failed to exhibit fluorescence. Permeabilized, chondroitinase ABC-treated mast cells incubated with the anti-keratan sulfate antibody also did not stain (Fig. 2*B*). No fluorescent staining was detected when the permeabilized cells were treated with large amounts of heparinase before the interaction with the anti-chondroitin sulfate monoclonal antibody. This excluded the possibility that a heparinase contaminant in the chondroitinase ABC preparation was creating a determinant from heparin that was recognized by the anti-chondroitin sulfate monoclonal antibody.

The specificity of the antibody-mediated fluorescence was also confirmed by absorption with solid-phase proteoglycans of an amount of 5/29/2-B-6 antibody that gave maximal, but just saturating, fluorescence staining of permeabilized, chondroitinase ABC-treated rat serosal mast cells. The relative amount of residual affinity-absorbed antibody was determined in each case by measuring its ability to mediate staining of rat serosal mast cells. When the 5/29/2-B-6 antibody was absorbed with chondroitinase ABC-digested chondrosarcoma chondroitin sulfate proteoglycan coupled to Sepharose, a 1:8 dilution of the absorbed antibody preparation stained only 3.7 ± 2.1% (mean ± S.D., $n = 3$) of the rat serosal mast cells (Fig. 3). Sixty-nine ± 11% (mean ± S.D., $n = 3$) of the cells stained after incubation with an identical dilution of unabsorbed 5/29/2-B-6 antibody. An equal amount of the 5/29/2-B-6 antibody that had been absorbed with non-chondroitinase ABC-treated proteoglycan and diluted 1:8 stained 57 ± 8.5% (mean ± S.D., $n = 3$) of the rat serosal mast cells, whereas a 1:8 dilution of antibody absorbed with heparin-agarose under identical conditions stained 70 ± 10% (mean ± S.D., $n = 3$) of the cells. Further dilution of the latter three antibody

preparations to 1:32 resulted in the staining of 35 ± 15%, 20 ± 5.7%, and 32 ± 17% (mean ± S.D., $n = 3$) of the cells, respectively, which was comparable to the activity of a 1:2–1:4 dilution of the monoclonal antibody following absorption with chondroitinase ABC-digested solid-phase chondroitin sulfate proteoglycan (Fig. 3).

DISCUSSION

Through the use of chemical and immunologic approaches, this study establishes that rat serosal mast cells contain chondroitin sulfate proteoglycans that are similar to the novel proteoglycans that can be ^{35}S-labeled *ex vivo* in rat mucosal mast cells. These highly sulfated chondroitin sulfate proteoglycans are rich in the disulfated disaccharides Di-diS_B (iduronic acid-2-SO_4→GalNAc-4-SO_4) and Di-diS_E (GlcA→GalNAc-4,6-diSO_4) (Fig. 1). Quantification of the chondroitin sulfate glycosaminoglycans by measurement of the unsaturated disaccharides released by chondroitinase ABC treatment of the density gradient-purified proteoglycans revealed that 10^6 rat serosal mast cells contain 2.4–4.5 μg of chondroitin sulfate proteoglycans, compared to a reported heparin proteoglycan content of more than 20 μg per 10^6 cells. Upon isolation and radiolabeling with [^{35}S]sulfate *in vitro*, rat serosal mast cells synthesize proteoglycans that contain heparin glycosaminoglycans but not chondroitin sulfate glycosaminoglycans (1), although biosynthesis of over-sulfated chondroitin sulfate can be observed in the presence of *p*-nitrophenyl-β-D-xyloside (9). Thus, either the rat serosal mast cell synthesizes chondroitin sulfate proteoglycans at an earlier stage of its differentiation or there is a preferential suppression of chondroitin sulfate synthesis when the cells are placed in short-term tissue culture.

Using a mouse IgG_1 monoclonal antibody prepared to chondroitinase ABC-treated bovine nasal cartilage proteoglycan, we determined by intracellular fluorescence microscopy that at least 91% of the permeabilized and chondroitinase ABC-treated cells in the rat serosal mast cell preparations contained chondroitin sulfate proteoglycans (Fig. 2*A*). The pattern of staining of the cells with the anti-chondroitin sulfate monoclonal antibody indicated that these proteoglycans were stored in the secretory granules of the cells. The *in situ* specificity of this monoclonal antibody for chondroitin sulfate glycosaminoglycans was limited to permeabilized, chondroitinase ABC-treated rat serosal mast cells (Fig. 2*A*). No fluorescence was observed in permeabilized, non-chondroitinase ABC-treated cells or in permeabilized, heparinase-treated cells. Solid-phase absorption of the antibody with chondroitinase ABC-digested chondroitin sulfate proteoglycan effectively abrogated subsequent binding to permeabilized, chondroitinase ABC-treated rat serosal mast cells (Fig. 3). However, solid-phase absorptions with equivalent amounts of either non-chondroitinase ABC-digested chondroitin sulfate proteoglycan or heparin did not reduce *in situ* binding to permeabilized and chondroitinase ABC-treated mast cells, as assessed by dilution analysis of absorbed antibody preparations compared with unabsorbed antibody (Fig. 3). Nonspecific absorption of IgG molecules and nonspecific binding of the secondary, fluorescein-conjugated antibody to the secretory granules were ruled out because the mouse IgG_1 monoclonal antibody that is reactive with keratan sulfate glycosaminoglycans did not bind to permeabilized and chondroitinase ABC-treated rat serosal mast cells (Fig. 2*B*). Although ~91% of chondroitinase ABC-treated rat serosal mast cells exhibited intracellular fluorescence when stained with the anti-chondroitin sulfate monoclonal antibody, there was considerable heterogeneity in the intensity of staining from cell to cell. This variation could be due to differential loss of the chondroitin sulfate proteoglycans during the permeabilization and enzymatic steps, to differences in the susceptibility of the chondroitin sulfate proteoglycans *in situ* to degradation by chondroitinase ABC because of varied interactions with other cationic proteins in the secretory granules, or it could reflect different stages of mast cell maturation.

The presence of both heparin and chondroitin sulfate glycosaminoglycans in highly purified, normal *ex vivo* serosal mast cells is a novel finding. In an earlier study we demonstrated that the rat basophilic leukemia-1 cell line, which by a number of criteria is homologous to rat mucosal mast cells (18), synthesizes both nitrous acid-degradable and chondroitinase ABC-degradable glycosaminoglycans (19, 20). As assessed by HPLC analysis of chondroitinase ABC-generated unsaturated disaccharides, the chondroitin sulfate of the rat basophilic leukemia-1 proteoglycan is composed of Di-4S and Di-diS_B (19). The presence of heparin and chondroitin sulfate that is rich in Di-diS_B, as well as Di-diS_E, in freshly isolated rat serosal mast cells demonstrates that the classification of subpopulations of rat mast cells by their proteoglycan phenotype may be more accurately defined by the ratios of the constituent glycosaminoglycans rather than by the absolute amount of a single glycosaminoglycan.

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