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MAST CELL EXOCYTOSIS: EVIDENCE THAT GRANULE PROTEOGLYCAN PROCESSING IS NOT COUPLED TO DEGRANULATION[1]

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It has been hypothesized that the dissolution of mast cell granules at the time of degranulation results from proteoglycan cleavage coupled to exocytosis. To address this hypothesis, we studied granule proteoglycan before and after exocytosis in dog mastocytoma cells, which solubilize granule contents during exocytosis. ^{35}S-labeled proteoglycans were extracted from unstimulated whole cells and cell degranulation supernatant. Sequential anion-exchange and gel filtration chromatography, followed by specific glycosaminoglycan digestion, identified chondroitin sulfate and heparin glycosaminoglycan and proteoglycan in unstimulated cells and degranulated material alike. Glycosaminoglycan type and charge density in degranulation supernatant were unchanged compared with unstimulated cells. There was no decrease in proteoglycan size with cell activation and exocytosis. Thus, granule release and solubilization does not appear to require exocytosis-coupled degradation of granule proteoglycans. Release in association with high-m.w. proteoglycans may serve to limit rates of diffusion and activity of proteases and other mast cell mediators.

Activated mast cells release the contents of their granules, which contain non-covalently associated proteases and PG (1-6), as well as other preformed mediators. Although classically considered to serve a packaging function, granule glycans, following release, also can modulate the behavior of mast cell proteases (7-12) and other proteins, including complement proteins (13) and growth factors (14). Degranulation of mast cells from different species and tissue sources is characterized by distinct differences in the solubility of granule-derived mediators following exocytosis. Rat peritoneal mast cells release granule contents in a complex which, with the exception of histamine, remains insoluble and largely intact (15), whereas human and dog mast cells release fully soluble PG and mediator proteins upon degranulation (5, 6, 16, 17). The differences in granule dissolution and mediator solubility may be biologically important, as they determine whether mediators such as the mast cell proteases remain confined to the immediate site of granule release or diffuse to reach distant targets.

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Abbreviations used: CHAPS, 3-(3-chlolamidopropyl)dimethylammonio-1-propanesulfonate; GAG, glycosaminoglycan; Gdn, guanidine; PG, proteoglycan.

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The mechanism underlying release of granule constituents has been the subject of speculation for some time. It has been proposed that cleavage of granule matrix is coupled to granule discharge, resulting in solubilization and liberation of granule components (18-20). In dog and human mast cells, which release fully soluble granule constituents, the issue of specific processing of granule PG at the time of cell activation and degranulation has not been addressed directly. In this study, we used dog mastocytoma cells, which solubilize their granule contents, to characterize granule PG isolated from unstimulated and activated mast cells to address the issue of PG processing in degranulation.

Materials and Methods

Materials. $Na_2{}^{35}SO_4$ (carrier-free, 25-40 Ci/mg) was obtained from New England Nuclear (Boston, MA). *Proteus vulgaris* chondroitin ABC lyase, Ca^{++} ionophore A23187, CHAPS, HEPES, guanidine (Gdn) HCl, thymol and carbohydrate aldonolactones were obtained from Sigma (St Louis, MO). Na borohydride and 6-aminohexanoic acid were from Aldrich Chemicals (Milwaukee, WI). Gdn HCl extraction buffer contained 4 M Gdn HCl, 0.15 M NaCl, 0.3% CHAPS, 0.1 M 6-aminohexanoic acid, 2 mM EDTA, 0.4 mM saccharo-1,4-lactone, 0.8 mM galactono-1,4-lactone, 0.1 N Na acetate (pH 5.8). Anion-exchange buffer contained 6 M urea, 1.0 M NaCl, 0.3% CHAPS, 0.1 N Na acetate (pH 5.8). Gel filtration buffer contained 0.5 M Gdn HCl, 0.2 M Na_2SO_4, 0.1 N Na acetate (pH 6.0), with 0.2% NaN_3.

Mast cell isolation and radiolabeling. Cells from the dog mastocytoma cell line BR, serially passaged in Balb/c mice (21), were harvested and disaggregated as described (22). Mast cells were incubated in HEPES-buffered Tyrode's medium (pH 7.4) containing 50 μCi/ml ^{35}S-sulfate, at 1.5 x 10^6 cells/ml for 16 h at 37°C. Dye exclusion studies demonstrated greater than 85% viability of cells after the labeling period. Cells were pelleted at 300 x g for 10 min, washed in Ca^{++}- and Mg^{++}-free Tyrode's medium, and subjected to PG extraction or to degranulation as described below.

Mast cell degranulation. Disaggregated BR dog mastocytoma cells, suspended at 3 to 5 x 10^6 cells/ml in Tyrodes buffer, were incubated with 5 μM ionophore A23187 for 20 min at 37°C, centrifuged at 300 x g for 10 min at room temperature, then chilled on ice for 10 min. ^{35}S-labeled molecules were then isolated from degranulation supernatant as described below. Independent experiments under the same degranulation conditions without radiolabel showed lactate dehydrogenase (LDH) release of less than 5% of total cellular LDH.

Extraction of ^{35}S-labeled material. Extractions from labeled and unlabeled cells were performed using the same protocol. For whole cells the cell pellet was suspended in 10 volumes of chilled Gdn HCl extraction buffer and sonicated with a Branson Model 350 Sonifier (Branson, Danbury, CT.), using the fine tip and lowest power setting, for 3 cycles of 10 s each, then extracted at 4°C for 18 h. Supernatant from ionophore-induced degranulation was added to 3 volumes of Gdn HCl extraction buffer and extracted at 4°C for 18 h. Material extracted from unstimulated cells and from degranulation supernatant was centrifuged at 40,000 x g for 50 min at 4°C, then exchanged into 6 M urea anion-exchange buffer using a G-25 Sephadex PD-10 column (Pharmacia LKB, Piscataway, N.J.). Samples were stored at -20°C.

Chromatography. Sonicated whole cell extracts or degranulation supernatant extracts were applied to an HR 5/10 Polyanion SI anion-exchange column (Pharmacia LKB) equilibrated in 6 M urea anion-exchange buffer. Samples were eluted in the same buffer with a 30 ml linear NaCl gradient of 1-3 M, collecting 1 ml fractions. Fraction aliquots were assayed for ^{35}S activity by scintillation spectrophotometry, and peaks were pooled according to scintillation counting results. Pooled material was dialyzed in 2000 D cutoff Spectropore dialysis tubing (Spectrum Medical Industries, Los Angeles, Ca.) against H_2O with thymol for 72 h at 4°C, lyophilized, and stored at -20°C. Samples of dialyzed anion-exchange peaks were dissolved in ~100 μl chromatography buffer, applied to a Superose 6 gel filtration column (Pharmacia LKB) equilibrated in the same buffer and eluted at 0.4 ml/min; 0.5 ml fractions were collected and an aliquot was used for liquid scintillation counting. Gel filtration of samples after nitrous acid or chondroitinase ABC cleavage were performed under the same conditions. Void volume (V_o) and total volume (V_t) for the column were determined using 2000 kD blue dextran and $^{35}SO_4$, respectively.

Glycan characterization. For identification of chondroitin sulfates, dialyzed and lyophilized aliquots of ^{35}S-labeled fractions from gel filtration were dissolved in 0.1 M Tris-acetate buffer (pH 8.0) containing 0.15 M NaCl, 40 mM NaF, and 0.05% BSA and digested with

chondroitinase ABC (0.05 units) for 16 h as previously described (22). An aliquot was incubated without enzyme as control. To identify heparin-containing ^{35}S-labeled glycans, samples were degraded with nitrous acid at pH 1.5 (22). Lyophilized products were dissolved in chromatography buffer for gel filtration on a Superose 6 column.

Results

Anion-exchange chromatography. Radiolabel was incorporated into BR mast cells with an efficiency of 0.4 to 2%. Degranulation released 40% of the total cell-associated radiolabel (average of two experiments). Anion-exchange chromatography of extracts of whole unstimulated cells and degranulation supernatant are compared in Fig. 1. The small peaks of radioactivity eluting in both chromatographic runs prior to the NaCl gradient were resistant to digestion by chondroitinase and by nitrous acid (data not shown) and exhibited absorbance at 280 nm, consistent with radiolabeled protein. Two well-resolved peaks were identified for each sample, with the early peak eluting at ~1.5 M NaCl and the late peak eluting at ~2.0 M NaCl. The early and late peaks from the whole cell extract (Fig. 1a) represented 10 and 90% of the total radiolabel eluted in the NaCl gradient, respectively. Corresponding peaks from degranulation supernatant (Fig. 1b) represented 11 and 89% of the total radiolabel, respectively. Fractions from peaks of both samples were pooled (Fig. 1) and dialyzed. Anticipating the late-eluting peak would contain heparin as well as a population of "oversulfated" chondroitin sulfates previously identified in BR cells (22), we divided the late-eluting peak into two pools (B_1 and B_2 in Fig. 1a) for separate analysis of GAG composition.

Gel filtration. Gel filtration chromatograms of ^{35}S-labeled material derived from unstimulated cell sonicates are shown in Fig. 2, with elution profiles of anion-exchange peaks A, B_1 and B_2 shown separately. For comparison with the corresponding gel filtration chromatograms derived from degranulation supernatant (Fig. 3), aliquots of pools B_1 and B_2 were combined and subjected to gel filtration (Fig. 2, bottom panel). The corresponding fractions A and B from anion-exchange of the degranulation supernatant sample (Fig. 1b) are shown in Fig. 3. Gel filtration of the early-eluting anion-exchange peak A samples from both sources reveals two peaks, with a high-m.w. peak in each sample having a K_{av} of ~0.23, and a lower m.w. peak with K_{av} of 0.85. The size characteristics of these high- and low-m.w. peaks suggest that they correspond to PG and GAG side chains, respectively. Gel filtration of samples from the late-eluting anion-exchange peak B of both unstimulated whole cells and degranulation supernatant reveals in each case a high-m.w.

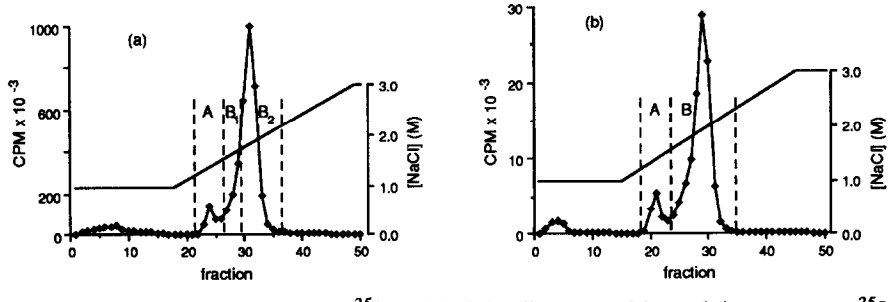

Figure 1. Anion-exchange chromatography of ^{35}S-labeled whole cell extracts and degranulation supernatant. ^{35}S-labeled molecules from unstimulated whole cells (a) and from degranulation supernatant (b) were eluted with a linear gradient of NaCl from an FPLC anion-exchange column. ^{35}S-activity peaks were divided into pools (A,B,B_1,B_2) as indicated by the vertical broken lines.

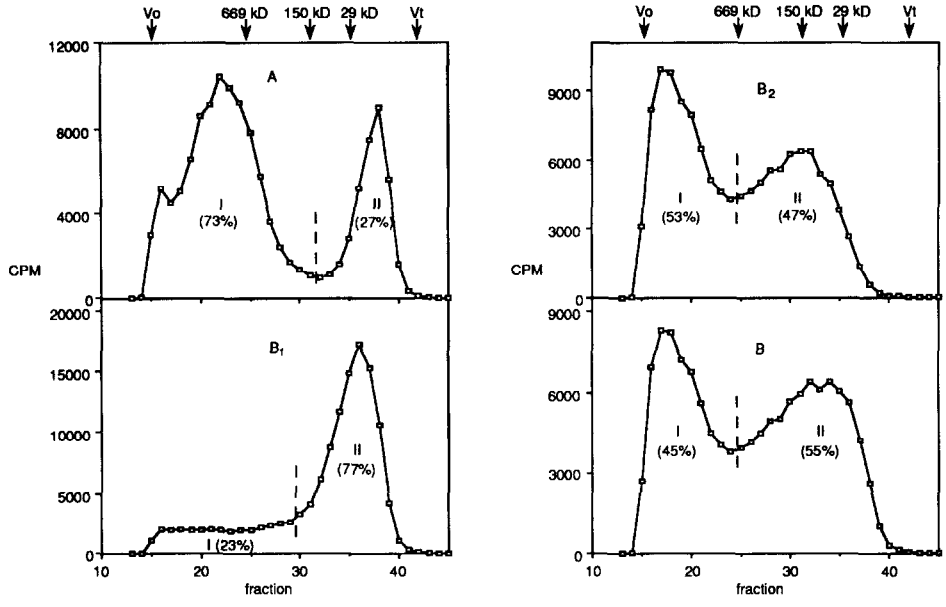

Figure 2. Gel filtration of anion-exchange peaks from whole cell extracts. Aliquots of pooled anion-exchange fractions from ^{35}S-labeled whole cell material (Fig. 1a) were subjected to Superose 6 gel filtration chromatography. The panels show the results (from top to bottom) of anion-exchange peak A, the B_1 and B_2 pools of anion-exchange peak B, and peak B recombined from the B_1 and B_2 fractions, respectively. Fractions were divided into pools I and II as indicated by the vertical broken lines, and aliquots were used for chondroitinase and nitrous acid digests. The percentage of total ^{35}S-activity for a sample which is contained in each peak is listed in parentheses in each panel. The elution positions for thyroglobulin (669 kD), alcohol dehydrogenase (150 kD) and carbonic anhydrase (29 kD) are indicated.

(K_{av} ~0.10) and a low-m.w. peak (K_{av} ~0.68). The B_1 fraction of the late-eluting anion-exchange peak from the unstimulated whole cell extract contains mainly low-m.w. material, as shown in Fig. 2. In contrast, the larger B_2 fraction of the late-eluting anion-exchange peak contains a predominance of high-m.w. material. Thus, the proportion of high- and low-m.w. species is distributed heterogeneously across the dominant, late-eluting anion-exchange peak derived from whole cells. The predominant glycan species in the early- and late-eluting anion-exchange peaks of extracts from both whole cells and degranulation supernatant is high m.w., consistent with PG.

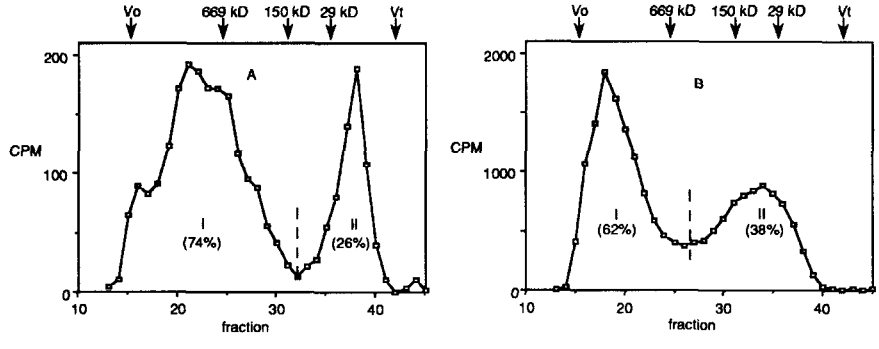

Figure 3. Gel filtration of anion-exchange peaks from degranulation supernatant extracts. Aliquots of pooled anion-exchange fractions from ^{35}S-labeled degranulation material (Fig.1b) were subjected to Superose 6 gel filtration chromatography. The left and right panels show the results of gel filtration of anion-exchange peaks A and B, respectively. The percentage of total ^{35}S-activity for a sample which is contained in each peak is listed in parentheses in each panel. Peaks were divided into two pools (I and II) as described in Fig. 2.

Most significantly, there is no shift to a higher K_{av} when comparing material derived from unstimulated whole cells with the ^{35}S-labeled molecules extracted from degranulation supernatant.

Chondroitinase ABC and nitrous acid cleavage. Aliquots of the high- and low-m.w. fractions from ^{35}S-labeled material initially separated by anion-exchange chromatography were subjected to digestion with chondroitinase ABC and nitrous acid. The resulting 20 digests (10 chondroitinase and 10 nitrous acid), plus controls were rechromatographed on a Superose 6 column. The results of gel filtration of nitrous acid and chondroitinase ABC digests are summarized in Table I, which lists the relative percent of ^{35}S activity due to chondroitin sulfate and heparin compared to total radioactivity in the unstimulated cell extract or in degranulation supernatant. In both whole cell material and degranulation supernatant, the lower ionic strength A peaks contain virtually all of their high-m.w. glycans (A-I) as chondroitin sulfate, while the lower m.w. peaks (A-II) are almost entirely heparin (presumably GAG). These results suggest that the early-eluting anion-exchange peaks contain chondroitin sulfate PG, which have been segregated by charge density from macromolecular heparin species. Heparin is the major GAG type identified in the material derived from the high ionic strength anion-exchange B peaks. However, the high-m.w. material in the B peaks from both whole cell extract and degranulation supernatant contains a significant amount of chondroitin sulfate in addition to heparin. 62% of the total chondroitin sulfate in the material from unstimulated cells and 72% of the total chondroitin sulfate in the degranulation supernatant are found in the high ionic strength peaks, suggesting that they may be "oversulfated" chondroitin sulfates with a charge density similar to heparin.

Overall, heparin is the dominant glycan in material extracted from whole cells as well as from degranulation supernatant (Table I). The total percentage of heparin from the two sources is similar (71 vs. 81%, respectively). There is no shift from high-m.w. PG to low-m.w. species associated with degranulation. Indeed, the percentage of large radiolabeled glycans is actually somewhat higher in the degranulation material. High-m.w. material constitutes 50% of the total in cell extracts and 63% of the total in degranulation supernatant. Heparin PG comprises 33% of the total ^{35}S-labeled material extracted from unstimulated cells compared to 35% of the total extracted from degranulation supernatant. Chondroitin sulfate PG, which is 17% of unstimulated cell

Table I

Heparin and Chondroitin Sulfate Composition for Whole Cell Extract and Degranulation Supernatant [a]

Whole cell extract			Degranulation supernatant		
fraction	% heparin	% chondroitin sulfate	fraction	% heparin	% chondroitin sulfate
A-I	0.2	7.2	A-I	0.1	8.0
A-II	2.6	0.1	A-II	2.8	0.1
B_1-I	1.9	2.3	B-I	35.3	19.9
B_1-II	13.6	0.3	B-II	32.1	1.0
B_2-I	30.5	7.6	total	71.0%	29.0%
B_2-II	32.1	1.7			
total	80.9%	19.1%			

(a) proportions are % of total ^{35}S activity for whole cells or supernatant

glycans and 27% of degranulation supernatant glycans, accounts for most of the difference between the two sources in the proportion of high-m.w. ^{35}S-labeled material.

The lower m.w. peaks (A-II and B-II) from whole cell and degranulation sources contain a predominance of heparin, with whole cell material showing a larger percentage of small m.w. species than in the degranulation supernatant (48.3% and 35.6%, respectively). Based on size, the glycans contained within the lower m.w. peaks consist mainly of free GAG chains.

Discussion

The most significant observation arising from our comparison of glycans from unstimulated cells and degranulated material is the lack of a shift from macromolecular forms to fragments during mast cell degranulation. This finding suggests that breakdown of macromolecular PG to low-m.w. forms is not necessary to achieve granule dissolution and solubilization of PG-bound mediators. Indeed, despite our calculation that ~63% of secreted ^{35}S-labeled glycans are high-m.w. PG, previous studies with these cells (6) indicate that the granules and their contents become fully soluble during release. Thus, other mechanisms to account for granule dissolution, such as the hydrolysis of structural granule constituents other than PG, or diminished molecular interactions due to a change in intragranular pH or ion and water flux, are likely.

Although we find no evidence to support degranulation-coupled processing of PG, there are differences between unstimulated cells and degranulation supernatant which deserve comment. The greatest difference in glycan distribution is in low-m.w. heparin, which in the unstimulated cells constitutes ~48% of the total ^{35}S-labeled material, while in the degranulation supernatant it is only ~35% of the total. Immature glycans present in presecretory organelles but absent in exocytosed material represent one potential explanation for this finding. Another difference between the two sources is the relative amount of ^{35}S-labeled chondroitin sulfates as a percentage of total radiolabeled material, which is 19.1% in whole cells and 29.0% in degranulation supernatant. Much of the increased percentage of chondroitin sulfates in the degranulation supernatant is due to an increased proportion of higher sulfation PG in fraction B-I (Table I). One possible explanation for this observation is that the releasable pool of chondroitin sulfate PG in mature granules is enriched in "oversulfated" forms compared to unstimulated whole cells, which may have a higher fraction of less mature chondroitins in presecretory organelles. To some extent, the higher percentage of chondroitin sulfate in the degranulation supernatant may be merely secondary to the lower proportion of low-m.w. heparin, as noted above. In any case, there is no observed shift from high- to low-m.w. forms of chondroitin sulfate associated with exocytosis; indeed, by far the dominant form of chondroitin sulfate from both unstimulated cells and degranulation supernatant is high-m.w., compatible with PG.

It has been argued that macromolecular PG, through ionic interactions, localize and limit the bioactivity of mast cell granule neutral proteases (23). PG breakdown could facilitate the diffusion of PG-bound preformed mast cell mediators, broadening their anatomic range of activity. Our evidence that PG cleavage does not accompany granule mediator release suggests that high-m.w. PG from solubilized granules may retain the capacity to limit granule protein diffusion and/or activity. However, our results also show that low-m.w. glycans are released with high-m.w. gly-

cans, even though PG cleavage does not appear to be linked to the process of degranulation itself. The heterogeneity of released glycan size, type and extent of sulfation may also lead to heterogeneity in the effects of glycans on the properties of neutral proteases such as inhibitor susceptibility, rates of diffusion, substrate targeting and rates of catalysis.

These results do not exclude *in vivo* degradation of granule PG. Indeed, there are reports of specific glycosidases that cleave PG or GAG (24). The early reports of glucuronidase activity associated with mast cells (25, 26) have been followed by more recent reports localizing this activity to macrophages (27). This potentially places the processing of macromolecular PG outside of mast cells. The presence of endoglycosidases in T-lymphocytes and neutrophils (28, 29), suggests the possibility that several varieties of leukocytes modulate biologic actions of secreted mast cell glycans.

In summary, dog mastocytoma cells known to release soluble preformed mediators upon degranulation were used to address the possibility that processing of granule PG is coupled to mast cell exocytosis. Compared to material extracted from unstimulated cells, soluble material released extracellularly upon mast cell degranulation contains high and essentially unaltered proportions of high-m.w. heparin and chondroitin sulfate glycans, suggesting that macromolecular PG breakdown is not essential for the release of soluble PG-bound mediators and that PG degradation is not linked to mast cell degranulation.

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