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Soluble NSF Attachment Protein Receptors (SNAREs) in RBL-2H3 Mast Cells: Functional Role of Syntaxin 4 in Exocytosis and Identification of a Vesicle-Associated Membrane Protein 8-Containing Secretory Compartment

Fabienne Paumet,* Joëlle Le Mao,* Sophie Martin,* Thierry Galli,† Bernard David,* Ulrich Blank,* and Michèlè Roa‡

Mast cells upon stimulation through high affinity IgE receptors massively release inflammatory mediators by the fusion of specialized secretory granules (related to lysosomes) with the plasma membrane. Using the RBL-2H3 rat mast cell line, we investigated whether granule secretion involves components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery. Several isoforms of each family of SNARE proteins were expressed. Among those, synaptosome-associated protein of 23 kDa (SNAP23) was central in SNARE complex formation. Within the syntaxin family, syntaxin 4 interacted with SNAP23 and all vesicle-associated membrane proteins (VAMPs) examined, except tetanus neurotoxin insensitive VAMP (TI-VAMP). Overexpression of syntaxin 4, but not of syntaxin 2 nor syntaxin 3, caused inhibition of FcεRI-dependent exocytosis. Four VAMP proteins, i.e., VAMP2, cellubrevin, TI-VAMP, and VAMP8, were present on intracellular membrane structures, with VAMP8 residing mainly on mediator-containing secretory granules. We suggest that syntaxin 4, SNAP23, and VAMP8 may be involved in regulation of mast cell exocytosis. Furthermore, these results are the first demonstration that the nonneuronal VAMP8 isoform, originally localized on early endosomes, is present in a regulated secretory compartment.

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1 Abbreviations used in this paper: SNARE, SNAP receptor; SNAP, soluble NSF attachment protein; GFP, green fluorescent protein; NEM, N-ethylmaleimide; NSF, NSF-sensitive factor; PE-SA, phycoerythrin-streptavidin; RBL-2H3, rat basophilic leukemia cell line; SNAP23, 25, 29, synaptosome-associated protein of 23 kDa, 25 kDa, 29 kDa; t-SNARE, target-SNARE; TI-VAMP, tetanus neurotoxin insensitive VAMP; V-SNARE, vesicle-SNARE; TRSC, Texas Red sulfonile chloride; VAMP, vesicle-associated membrane protein.
Although the bulk of available information on SNARE proteins concerns neuronal and neuroendocrine cells, these proteins are also implicated in the regulated exocytosis of cells of the hemopoietic lineage (39, 40). In mast cells, the expression of several SNARE proteins has been reported, and SNAP23 relocation was shown to be coupled to secretion in permeabilized cells (41, 42). The aim of our study was to characterize additional SNARE proteins expressed in RBL-2H3 mast cells and to investigate their role in FcεRI-triggered exocytosis. We show that besides SNAP23, syntaxin 4 is the only t-SNARE that interacts with the identified v-SNAREs in intact cells. We provide evidence for the function of syntaxin 4 in degranulation. Furthermore, we observe that VAMP8 is tetanus toxin insensitive and colocalizes with a part of the secretory granules, suggesting this v-SNARE as the potential partner of syntaxin 4 and SNAP23 in the regulated secretion of mast cells.

Materials and Methods

Cell cultures, stimulation, and secretion assay

RBL-2H3, COS-7, and transiently transfected cells were maintained as monolayer cultures, as previously described (37). For stimulation, RBL-2H3 cells were seeded in 175-mm² flasks (70% confluence) or used in suspension (1 × 10⁶ cells/ml), sensitized with IgE anti-DNP, and stimulated with Ag, as described (37).

RT-PCR and PCR

Total RNA was isolated as described (37). For RT-PCR, 2 µg of total RNA of RBL-2H3 cells or brain was reverse transcribed using Superscript reverse transcriptase (Life Technologies, Eragny, France). The cDNAs were amplified between bp 13 and 544 for SNAP25a/b (GenBank database accession number U56261/U56262), 1 and 633 for SNAP23/syn3dynt (UT3143), 245/162 and 404/321 for VAMP1/VAMP2 (M24104/M24105), 104 and 415 for cellubrevin (S68380), 67 and 290 for VAMP8 (AA049140), 1 and 874 and 1 and 794 for, respectively, full-length and truncated syntaxin 2 (L20823), and 32 and 928 and 328 and 841 for, respectively, full-length and truncated syntaxin 4 (L20821). The cDNAs for syntaxin 3 were obtained from Dr. R. H. Scheller (Stanford University, School of Medicine, Stanford, CA) and were amplified between bp 78 and 947 and 78 and 866 to generate, respectively, full-length and truncated cDNAs (L20820).

cDNA constructs and recombinant proteins

Truncated and full-length cDNAs for syntaxin 2, 3, and 4 were cloned in the eukaryotic expression vector pSRαpuro (37). pEGFP-C1 vector was purchased from Clontech (Palo Alto, CA). The cDNA for RFcoα was a gift of Dr. R. Monteiro (Hôpital Necker, Paris, France). The cytoplasmic domains of syntaxin 4a265 (syn4AC), syntaxin 3a265 (syn3AC), and syntaxin 4b290 (syn4LC) were also expressed as GST fusion proteins using the pGEX-2TK vector (Amersham Pharmacia Biotech, Uppsala, Sweden). Fusion proteins were purified as described in the supplier’s protocols. Thrombin (Boehringer Mannheim, Indianapolis, IN) was used to release the syntaxins from GST. All cDNAs concerning syntaxin isoforms were sequenced and compared with the corresponding data deposited in GenBank.

Antibodies

New Zealand white rabbits were immunized with the cytoplasmic domains of rat syntaxin 2, syntaxin 3, and syntaxin 4 proteins. Abs were affinity purified from sera on Sepharose 4B (Amersham Pharmacia Biotech) coupled to GST-syn2ΔC, GST-syn3ΔC, and GST-syn4ΔC fusion proteins, respectively. Each batch was further depleted of common syntaxin epitopes using serial Sepharose 4B columns coupled to the two other cross-reacting isoforms. Affinity-purified anti-GST Abs (our laboratory) have been used. Anti-DNP-specific IgE and FcεRI β-chain mAbs, IRX, provided by Dr. J. Rivera (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD), have been described (37). Rabbit anti-SNAP23 (TG8), anti-VAMP1 (MC9), anti-cellubrevin (MC16) sera, as well as affinity-purified anti-TI-VAMP (TG11), and anti-VAMP8 (TG15) Abs have been used (22, 30, 32). Anti-syntaxin 1 mouse mAb MC16 from Sigma (St. Louis, MO). Rabbit anti-VAMP2 Abs (43) were provided by A. Klip and B. Trimble (The Hospital for Sick Children, Toronto, Canada). The mouse anti-serotonin mAb (clone 5HT- H209) was from Dako (Trappes, France). Mouse mAbs against rat p80 Ag and against human CD8 were produced respectively from the 5G10 clone provided by Dr. J. Bonifacino (44) and from the 10D11.5 clone. Both Abs were biotinylated with the Enzotin Biotinylation Kit, according to the manufacturer’s instructions (EnzBiochem, Syosset, NY). Biotinylated mouse mAb A59 directed against RFcoα was provided by Dr. R. Monteiro. Peroxidase-coupled goat anti-rabbit and goat anti-mouse IgGs and Texas Red sulfonide chloride (TRSC)-conjugated goat anti-mouse IgG, all specific for the Fcγ fragment, were purchased at Jackson Immunoresearch (West Grove, PA).FITC-conjugated goat anti-rabbit IgGs (H + L) was from Biosys S.A. (Compiègne, France).

Confocal immunofluorescence microscopy

RBL-2H3 cells were seeded on 10-mm-diameter glass coverslips (2 × 10⁶ cells/coverslip) 16 h before analysis. For indirect immunofluorescence analysis, cells were fixed in 3% paraformaldehyde for 10 min, followed by treatment with 50 mM NH₄Cl for 15 min. After washing, cells were permeabilized in 0.05% saponin and saturated in the presence of 0.25% BSA and 5% goat serum (MedGene Science, Pantin, France). Primary Abs were incubated 1 h in the same buffer and then revealed with FITC- and/or TRSC-conjugated secondary Abs. Finally, the coverslips were mounted in Mowiol and viewed with a Leica TCS confocal laser-scanning microscope.

NEM treatment

For some experiments, rat brain and RBL-2H3 homogenates were extracted with TCA, as described (45). For immunoprecipitation, RBL-2H3 cells (5 × 10⁶ cells/ml) were lysed on ice for 1 h in buffer A (50 mM Mops, pH 8, 150 mM NaCl, 10 mM EDTA, and 1% Triton X-100) containing a mixture of protease inhibitors. After centrifugation for 30 min at 14,000 × g, soluble lysates were incubated overnight at 4°C with Abs prebound to protein A-Sepharose beads (Amersham Pharmacia Biotech). Beads were pelleted and washed three times with buffer A and once with buffer A 0.5% Triton X-100. Immunoprecipitated complexes were resolved on SDS-PAGE gels (46). Experimental conditions for transfer to nitrocellulose membrane, Ab incubations, and enhanced chemiluminescent (ECL) revelation were as described (37).

Transfections

Transient transfections into COS-7 cells were as reported (37). For functional secretion assays, a transient cotransfection procedure was optimized for RBL-2H3 cells. Briefly, 4 × 10⁶ cells kept at room temperature were electroporated using 5 µg of vector pEGFP-C1 (transfection marker) and 1 µg of respectively pSRαpuro-syntaxin 2, pSRαpuro-syntaxin 3, pSRαpuro-syntaxin 4, or pSRαpuro (empty vector) in complete DMEM medium. The cells received a single electrical pulse of 250 V, 1500 microfarads, using an Eurebio EasyJect electroporation apparatus (Eurogentec, Angers, France). Cells were then plated in complete medium, which was replaced after 24 h. Exocytosis was determined 48 h after transfection using 5G10 mAb (see below). Using these conditions, the efficiency of transfection (GFP-positive cells) was routinely in the range of 30% of total population, as determined by flow cytometry (see below). The co-transfection efficiency was estimated to be over 80%, as tested by cotransfection of GFP and RFcoα cDNAs, the latter being detected at the surface of cells cotransfected with A59 mAb and PE-SA (Dako).

Exocytosis measurement by flow cytometry

Transfected RBL-2H3 cells in suspension were sensitized with IgE for 2 h and stimulated for 20 min. After washing in cold PBS-0.05% BSA (buffer B), all pellets were incubated on ice for 1 h with 20 µg/ml biotinylated 5G10 or 10D11.5 mAb. After washing, the cells were reincubated for 1 h at 4°C with PE-SA in buffer B, washed again, and resuspended in 0.5 ml cold buffer B for analysis in a FACScan cyttofluorograph (Becton Dickenson, San Jose, CA). Fluorescence of 50,000 viable cells was analyzed, and GFP-positive cells were selected within a gate on the FL1 channel. Fifty Abs were selected and used to identify rabbit anti-VAMP2 Abs was measured by p80 exposure at cell surface. Exposure of this Ag and secretion are closely correlated in time and Ag dose response (Ref. 44 and our unpublished results). In our conditions, maximal values of degranulation were obtained

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after 20 min and were in the range of 30–50% of total β-hexosaminidase content. Expression of p80 Ag was measured using biotin-5G10 plus PE-SA in the FL2 channel (585/42 band path filter). Biotin-10D11.5 was used as an irrelevant Ab to detect nonspecific binding. Data were analyzed using CellQuest software, and expression levels were quantified by calculating the ratio of the mean fluorescence intensities of specific (5G10) vs irrelevant (10D11.5) mAb staining. All data were analyzed by using the Mann-Whitney U test. Significance was assumed at p values < 0.05.

**Tetanus toxin proteolysis**

RBL-2H3 cells (1.6 × 10⁶ cells/ml) were lysed at 4°C in 150 mM NaCl, 8 mM MgCl₂, 300 mM glycine, and 20 mM HEPES, pH 7.4, containing a mixture of protease inhibitors and sonicated three times 10 s in ice. After removing the debris at 14,000 g for 1 min, 30-μl aliquots were incubated for 2.5 h at 37°C with various concentrations of light chain tetanus toxin (a gift of Dr. M. Popoff, Institut Pasteur, Paris, France). This chain bears the catalytic activity of the toxin. Proteolysis was stopped by addition of sample buffer under reducing conditions and boiling for 3.5 min.

**Results**

**SNARE proteins are endogenously expressed in RBL-2H3 cells**

In initial experiments, mRNA expression in RBL-2H3 for different t- and v-SNAREs was assessed by RT-PCR, taking rat brain mRNA as a control. In the case of syntaxins, in which multiple isoforms have been characterized, we particularly searched for those that have been described to be present on the plasma membrane. We identified a large number of expressed SNARE mRNAs. They included the t-SNAREs syntaxin 2, syntaxin 3, syntaxin 4, and SNAP23, as well as the v-SNAREs VAMP1/2, cellubrevin, and VAMP8. No SNAP25 mRNA was found (data not shown). All fragments amplified by RT-PCR corresponded to the expected size; in addition, the DNA sequences for syntaxins 2, 3, and 4 were verified.

To characterize t- and v-SNAREs, we examined expressed proteins by immunoblot analysis. We produced Abs against the three syntaxin isoforms identified above. Their isoform specificity was tested by immunoblot analysis of COS-7 cells transfected with cDNAs coding for either syn2ΔC, syn3ΔC, syn4ΔC, or empty pSRα vector (mock) were resolved on SDS-PAGE and immunoblotted with anti-syntaxin 2, 3, and 4 Abs. Bands were visualized using ECL for detection.

**SNAP23 and detected a band migrating at about 29 kDa in RBL-2H3 extracts and in the brain.** Concerning v-SNAREs, VAMP2 (18 kDa) and cellubrevin (14 kDa), but not VAMP1, were expressed in RBL-2H3 cells (Fig. 2B). In addition, we revealed VAMP8, a newly described isoform of 12 kDa, and a faint band of 25 kDa corresponding to TI-VAMP. The above results show that isoforms of each protein family composing the SNARE core complex are expressed in RBL-2H3 mast cells, thus confirming and extending recent data obtained in rat peritoneal mast cells (42). To further explore these findings, we investigated their interactions in intact cells and their possible involvement in degranulation.

**Plasma membrane SNAP23 is central in SNARE complexes in RBL-2H3 cells**

Because the t-SNARE SNAP23 has been shown to play a role in exocytosis of rat peritoneal mast cells (42), we analyzed its subcellular location in RBL-2H3 cells and tested its capacity to form SNARE complexes. The staining pattern observed by confocal immunofluorescence microscopy was shown in Fig. 3A. A bright staining was uniformly distributed on the plasma membrane, no signal being visible in intracellular organelles. To characterize the potential partners associated with SNAP23, a commnunoprecipitation approach was undertaken. As protein interactions were very faint in resting cells, we made use of NEM, a sulfhydryl-alkylating agent known to inactivate NSF (47) and induce SNARE complex accumulation (32, 48). After NEM treatment of RBL-2H3 cells,
we immunoprecipitated SNAP23 and screened for the interactions with syntaxin and VAMP proteins using immunoblotting (Fig. 3B). As expected, all interactions were increased after NEM treatment. SNAP23 was able to form multiple interactions with syntaxin 2, syntaxin 3, and syntaxin 4, although communoprecipitation of syntaxin 3 was very faint even in the presence of NEM. Interactions were also detectable with VAMP isoforms VAMP2, cellubrevin, and VAMP8, but not TI-VAMP (data not shown). Taken together, these results show that SNAP23 is capable of forming different sets of SNARE complexes in RBL-2H3 cells.

**Only syntaxin 4 interacts with a specific set of SNARE proteins**

As SNAP23 seems to be present in multiple complexes with different syntaxin isoforms, we examined more precisely the role of this second group of t-SNARE proteins. Syntaxins 2, 3, and 4 were immunoprecipitated from cell lysates before and after NEM treatment, and the presence of associated proteins was investigated by immunoblotting (Fig. 4). In syntaxin 2 and 3 immunoprecipitates, SNAP23 was only seen after treatment, and none of the examined v-SNAREs was revealed. In contrast, syntaxin 4 immunoprecipitates contained SNAP23 as well as VAMP2, cellubrevin, and VAMP8, whereas TI-VAMP was undetectable (not shown). Thus, in addition to SNAP23, syntaxin 4 may be a good candidate for the formation of SNARE complexes, ensuring the traffic between granules and the plasma membrane.

**Syntaxin 4 is functionally involved in FcεRI-stimulated degranulation**

We investigated in a next step whether the overexpression of syntaxin 4 could affect FcεRI-mediated degranulation compared with overexpression of syntaxins 2 and 3. For this purpose, we developed an assay that allows the quantitative analysis of exocytosis of single cells using flow cytofluorometry. This assay took advantage of the stimulation-dependent surface expression of the intragranular protein p80 to measure exocytosis. The change of localization has been shown to correlate with the release of granular content (44). RBL-2H3 cells were transiently transfected with the cDNA coding for each syntaxin or empty vector together with GFP as a marker for the transfected population. We verified that each syntaxin was overexpressed using isoform-specific syntaxin Abs (Fig. 5A). Cells were then stimulated with Ag, and p80 expression was measured by cytofluorometry after gating GFP-positive cells. Fig. 5B illustrates the distribution of fluorescence intensity for each type of transfectant in a typical experiment. The histogram for syntaxin 4 transfectants shifted to lower levels as compared with those for syntaxin 2, 3, or mock-transfected cells, indicating a decrease in p80 levels at the surface after overexpression of syntaxin 4. The mean inhibition of p80 expression for each type of transfectant is presented in Fig. 5C. Overexpression of syntaxin 4 significantly inhibited p80 expression by 31% (p = 0.0039). In contrast, overexpression of syntaxin 2 and syntaxin 3 did not affect p80 expression when compared with mock-transfected cells (p > 0.5). In conclusion, our results strongly support a role for syntaxin 4 in regulated exocytosis of RBL-2H3 cells.

**VAMP8 is insensitive to tetanus toxin**

Because SNAP23 and syntaxin 4 appear to be part of the SNARE core complex involved in RBL-2H3 exocytosis, we investigated which VAMP isoform(s) could constitute the third member of this complex. A particular feature of VAMP2 and cellubrevin is their sensitivity to tetanus toxin (29, 49). Previous functional studies have shown that RBL-2H3 degranulation is resistant to this toxin, suggestive of a role for a tetanus toxin-insensitive VAMP isoform in that process (50). Fig. 6 confirms that VAMP2 and cellubrevin were sensitive to tetanus toxin in a dose-dependent manner, while
TI-VAMP was resistant in RBL-2H3 lysates. Interestingly, VAMP8 was also insensitive to this neurotoxin even at concentrations as high as 0.45 μM. These results point to TI-VAMP and VAMP8 as candidates for being part of the exocytotic process in RBL-2H3 cells.

VAMP8 is localized on secretory granules

To further investigate the role of these v-SNAREs, we examined the subcellular localization of VAMP proteins in RBL-2H3 resting cells. VAMP2 and cellubrevin both stained diffuse small vesicular structures (Fig. 7, A and B), whereas TI-VAMP and VAMP8 stained larger organelles distributed throughout the cytoplasm (Fig. 7, C and D). Colocalization experiments were performed using a mAb directed against serotonin, which specifically labels mast cell secretory granules. In agreement with their earlier described localization (51), serotonin-containing compartments appeared as large granular organelles (Fig. 7E–H). It should be noted that not all cells are serotonin positive, possibly reflecting a difference in their stage of maturation. There was no significant colocalization of VAMP2 or TI-VAMP with serotonin-containing structures (compare A and E, C and G, respectively, and overlay images I and K), and only a limited colocalization of cellubrevin with these serotonin-containing structures. On the contrary, VAMP8 colocalized with a larger set of the serotonin-containing vesicles, suggesting that VAMP8 is present on a subset of secretory granules (D, H, and overlay in L). Altogether, these results...
could support the involvement of VAMP8 in exocytosis of these mediator-containing granules.

Discussion
Although many unique SNAREs with specific locations have been identified, we are only beginning to understand how these proteins regulate the specificity of particular vesicle-mediated transport pathways. The hemopoietic cell lineage, with the rather peculiar feature of secretory lysosomes, may use specialized mechanisms for sorting and secretion, which differ from those found in conventional secretory cells (7). Taking RBL-2H3 cells as a model for mast cells, we show that several isoforms of the three SNARE protein families are expressed and that they form different sets of complexes in intact cells. We provide evidence for a functional role of syntaxin 4 in FcεRI-stimulated secretion and for the localization of its binding partner, the v-SNARE VAMP8, on secretory granules.

Identification of t-SNARE proteins SNAP23, syntaxin 3, and syntaxin 4 as well as the v-SNARE VAMP2 corroborates previous observations in rat peritoneal mast cells (42). However, in adherent RBL-2H3 cells, SNAP23 is linearly localized along the plasma membrane at discrepancy with the patchy appearance of SNAP23 reported in suspended peritoneal mast cells (42). A distinct functional state of the cellular cytoskeleton according to the status of adherence of the mast cell could explain this difference (52). Indeed, SNAP23 may interact with the cytoskeleton, possibly actin microfilaments, as reported in suspended peritoneal mast cells (42). The characterization of syntaxin 2, cellubrevin, TI-VAMP,
and VAMP8 reported in this work further extends the number of SNARE proteins potentially involved in regulation of intracellular traffic in mast cells.

SNARE proteins function in membrane fusion by cognate pairing to form a stable receptor for SNAP and NSF proteins. In resting RBL-2H3 cells, we detected a small amount of SNAP23, VAMP2, or cellubrevin in interaction with syntaxin 4. Following NEM treatment, which is known to block NSF and accumulate SNARE complexes, all of these interactions were increased, and those between syntaxin 4 and VAMP8 could be detected. On the contrary, neither syntaxin 2 nor syntaxin 3 was able to bind these different SNAREs, except a faint binding with SNAP23. Interestingly, both syntaxins are found in intracellular organelles in RBL-2H3 cells (Ref. 42 and our unpublished results), suggesting that they are implicated in other trafficking pathways remaining to be defined, possibly related to the various types of granules present in these cells. Altogether, our coimmunoprecipitation results support the idea that syntaxin 4 as well as SNAP23 preferentially interact with VAMP2, cellubrevin, and VAMP8 in intact cells.

The implication of syntaxin 4 in degranulation is confirmed by functional studies. Indeed, overexpression of syntaxin 4, but not of syntaxin 2 nor 3, significantly inhibited FcεRI-mediated exocytosis. A role for syntaxin 4 in regulating vesicular docking and fusion events has also been reported in α-granule secretion of platelets (39) and in adipocytes (53), suggesting that syntaxin 4 is an important t-SNARE in regulated exocytosis of nonneuronal cells. The mechanisms by which overexpression of wild-type syntaxin 4 inhibits regulated exocytosis pathways is not completely clear. Overproduction of this syntaxin protein may perturb the steady state between the three SNARE, components leading to aberrant interactions and blockade of recycling by “tittering” general factors like NSF or SNAP proteins. Alternatively, overexpression of syntaxin 4 could disturb regulatory factors implicated in the specificity of SNARE complexes such as munc18 isoforms. Two lines of evidence argue in favor of that hypothesis: 1) overexpression of a munc18 homologue involved in endoplasmic reticulum to Golgi transport was able to overcome the dominant negative effects of overexpressed syntaxin 5 (54); and 2) munc18 expression together with syntaxin 1A partially relieves the blockage of secretion in a storage compartment observed when expressing syntaxin 1A alone (55). A third possibility relates to Rab GTases, which, among the proteins playing a key role in the secretory pathway, could facilitate SNARE complex formation (56, 57). Rab3d, involved in mast cell exocytosis, (37, 58), may be a good candidate for direct or indirect interactions with syntaxin 4.

The function of this t-SNARE in exocytosis implies the mobilization of syntaxin 4-containing complexes during stimulation. However, we were not able to show that any of the proteins interacting with syntaxin 4, i.e., SNAP23, VAMP2, cellubrevin, or VAMP8, were modulated in response to FcεRI-mediated stimulation (data not shown). These observations either suggest that there is no change or that this change is too subtle to be revealed by coimmunoprecipitation experiments. In favor of the latter hypothesis, a kinetic analysis of SNARE complexes in chromaffin cells has revealed a short lifetime of complexes, suggesting that they cannot be stabilized physiologically in sufficient amounts (59, 60). In addition, mast cells possess a particular mechanism of granule fusion known as compound exocytosis, which is characterized by the fusion of adjacent granules with each other before fusion with plasma membrane (61, 62). This peculiar feature may explain the very weak interaction between syntaxin 4 and other SNARE proteins even after Ag-dependent stimulation, because in this process only few interactions between the plasma membrane and pre fused granules are necessary to allow massive granular discharge.

Concerning v-SNAREs involved in exocytosis, two independent findings could support for a role for VAMP8. On the one hand, we have demonstrated that VAMP8 was colocalized, at least in large part, with serotonin-containing granules, in contrast to the other VAMPs examined (cellubrevin was found to be superimposed with serotonin in a small subset of structures). On the other hand, we have found that VAMP8 was insensitive to tetanus toxin treatment, the latter result being consistent with the data showing that RBL-2H3 cells pretreated with this neurotoxin (50).

The localization of VAMP8 to a subset of secretory granules is a new finding in light of previous data that have shown its enrichment in the later compartment of early endosomes for a number of cell types (34). This particular staining pattern could be characteristic for the lysosomal nature of these organelles in mast cells (4, 5, 7). Indeed, recent data have demonstrated the existence of at least three types of secretory granules, all of them containing the lysosomal enzyme β hexosaminidase (3, 38). Type I and type II granules contained MHC class II molecules and were labeled by fluid endocytic markers, whereas serotonin was only observed in type II and type III granules. Given its endocytic nature, an attractive hypothesis would be to consider VAMP8 as a marker for type I and type II granules. The latter would be consistent with the observed staining pattern, because VAMP8 labels both serotonin-negative (which would correspond to type I) and part of the serotonin-positive (which would correspond to type II) granules. VAMP8 localization on serotonin-containing granules could thus represent a specific feature of mast cells, or more widely, of secretory cells within the hemopoietic cell lineage.

In conclusion, the present study demonstrates that the SNARE molecular machinery is involved in RBL-2H3 degranulation, and suggests that it depends on an exocytotic complex composed of syntaxin 4, SNAP23, and VAMP8. The presence of VAMP8 in this complex opens the possibility that a v-SNARE, originally localized on an endosomal compartment, functions in exocytosis. In this context, secretory granules in mast cells could be considered as a specialized compartment at the intersection between endocytic and exocytotic pathways.

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