

Original article

A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils

Background: Granulocyte exocytosis is proposed to be critically dependent on the interaction of soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) located on granules/vesicles (v-SNAREs) and plasma membrane (t-SNAREs). Previous studies indicated that the v-SNARE, vesicle-associated membrane protein (VAMP)-2, as well as t-SNAREs (SNAP-23, syntaxin-4 and -6) are implicated in exocytosis from human granulocytes. Vesicle-associated membrane proteins-7 and -8 have been implicated in endosome/lysosome trafficking, however, their role in granulocyte exocytosis remains obscure.

Objective: We sought to investigate the expression and functional role of SNARE isoforms in the secretion of different granule-derived mediators in human eosinophils and neutrophils.

Methods: The expression of SNAREs was determined by subcellular fractionation and flow cytometry. SNARE-specific antibodies were examined for their ability to impair mediator release from permeabilized eosinophils and neutrophils.

Results: Vesicle-associated membrane proteins-7 and -8 were localized to granule and membrane-enriched fractions in eosinophils and neutrophils, whereas syntaxin-6 was not detectable. In permeabilized cells, anti-VAMP-7, but not anti-VAMP-8, antibody impaired the secretion of all mediators examined (in eosinophils, eosinophil peroxidase and eosinophil-derived neurotoxin; in neutrophils, myeloperoxidase, lactoferrin and matrix metalloproteinase-9) in a dose-dependent manner. In contrast, anti-VAMP-2 modestly and selectively impaired secretion from small granules and vesicles. Syntaxin-4, but not syntaxin-6, was found to interact with SNAP-23 and was partially involved in mediator secretion from multiple compartments.

Conclusion: Our observations indicate for the first time a critical role for VAMP-7 in both eosinophil and neutrophil mediator release.

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The release of preformed granule-derived mediators from eosinophils and neutrophils is critical in the manifestation of inflammatory responses in airway diseases such as asthma and chronic obstructive pulmonary disease (1, 2). Eosinophils exhibit a rapid mobilization of secretory vesicles, termed piecemeal degranulation, which may or may not coincide with large crystalloid granule exocytosis depending on culture conditions or activating agents (3, 4). Neutrophils have four distinct granules: azurophilic granules, small (secondary) granules, tertiary granules and secretory vesicles. Similar to eosinophils, mediator release from neutrophils is characterized by a rapid and sequential release of secretory vesicles, secondary granules and/or tertiary granules, followed by the slower release of azurophilic granules (5).

Exocytosis of membrane-bound vesicles/granules has been shown to be critically dependent on the interaction of one vesicle (v-) soluble *N*-ethylmaleimide-sensitive

factor attachment protein (SNAP) receptors (SNARE) isoform, vesicle-associated membrane protein (VAMP), with two isoforms of target (t-) SNAREs: a syntaxin isoform and either synaptosome-associated-protein of 25 or 23 kDa (SNAP-25 or SNAP-23) (6, 7). It has been hypothesized that distinct SNARE isoforms may, in part, determine the specificity of vesicle/granule trafficking. In accordance with this paradigm, it may be anticipated that different granule populations express nonoverlapping sets of VAMPs. However, studies have supported the promiscuous interactions of SNAREs *in vitro* (8) and that single SNAREs are capable of participating in multiple trafficking steps *in vivo* (7).

We previously showed VAMP-2 was localized to secretory vesicles, but not on crystalloid granules, in human eosinophils and implicated in IFN- γ -induced exocytosis (9). In separate studies, VAMP-2 was implicated in exocytosis of neutrophil secretory vesicles,

secondary and tertiary granules (10). Both human eosinophils (11) and neutrophils (12) express the t-SNAREs, SNAP-23 and syntaxin-4, which are localized, although not exclusively, to plasma membranes. In addition, syntaxin-6 has been implicated in exocytosis of neutrophil mediators (12). Vesicle-associated membrane protein(s) localized to eosinophil crystalloid granules and neutrophil azurophilic granules have not been identified. Recent investigations indicated that the isoforms, VAMP-7 and -8, are involved in endocytic and/or exocytic trafficking (13–16). In this study, we sought to determine whether there is a differential role of v-SNAREs (VAMP-2, -7 and -8) in the release of compartment-specific stored mediators. We also examined the functional role of syntaxin-4 and -6, previously shown to be involved in human neutrophils (10, 12), in the release of multiple granule-derived mediators. Our data demonstrate, for the first time, a critical role for VAMP-7 in the secretion of stored mediators from multiple granule populations in human granulocytes.

Methods

Materials

Anti-human syntaxin-4, anti-rat syntaxin-6 and anti-CD63 monoclonal antibodies (mAbs; immunoglobulin G₁ (IgG₁; BD Pharmingen, San Diego, CA). Anti-VAMP-2 mAb (Cl 69.1) and rabbit anti-SNAP-23 (Synaptic Systems GmbH, Göttingen, Germany). Anti-human VAMP-7 mAb (clone 158.2) (17) and human VAMP-8 (TG15) rabbit IgG (18) were gifts from Dr T. Galli (INSERM, Paris, France). Mouse IgG₁ (mIgG₁; R&D Systems, Minneapolis, MN) and purified rabbit-IgG (Cedarlane Laboratories, Hornby, ON, Canada). Anti-human-major basic protein (MBP) (BMK-13) mAb was described previously (19). Streptolysin-O (SLO) was purchased from Dr S. Bhakdi (Johannes Gutenberg University, Mainz, Germany). Botulinum B-light chain was purchased from Calbiochem (La Jolla, CA).

Preparation of eosinophils and neutrophils

Eosinophils and neutrophils from atopic and healthy individuals, respectively, were isolated as described previously (20). Briefly, erythrocytes from peripheral blood (50 or 100 ml) were sedimented in 6% dextran, followed by Ficoll (Amersham Biosciences, Oakville, Canada) centrifugation with resulting neutrophil purity >98%. CD16⁺ eosinophils (>99%) were isolated by negative immunomagnetic selection (Miltenyi Biotec Inc., Auburn, CA).

Subcellular fractionation

Subcellular fractions of $5\text{--}8 \times 10^7$ eosinophils or neutrophils were separated on a linear Nycodenz gradient as described previously (11) and examined for marker proteins: eosinophil peroxidase (EPO) and myeloperoxidase (MPO) (eosinophil crystalloid granules and neutrophil AG respectively), β -hexosaminidase (eosinophil crystalloid granules) and lactate dehydrogenase (cytoplasmic marker). Membrane-enriched fractions were detected by CD9 dot-blot analysis for eosinophils and alkaline phosphatase for neutrophils.

Concentrated high-speed membrane pellets were generated by high speed (100 000 *g*) centrifugation as previously described (11).

Western blot analysis and immunoprecipitation

Subcellular fractions were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (25 μ l/lane) and Western blot analysis with positive controls: human platelets, rat brain and CaCo-2 cells. CaCo-2 cells were provided by Dr Glen Armstrong (University of Calgary). For co-precipitation experiments, membrane fractions were pelleted by centrifugation for 1 h at 100 000 *g*, resuspended in 0.5 ml PBS + 1% Nonidet P-40 + 0.1% SDS containing 5 μ g/ml protease inhibitors [leupeptin, aprotinin and TAME (Sigma, Oakville, ON, Canada)], precleared with 2.0 μ g mouse IgG₁ and 20 μ l of Protein G Sepharose (Amersham Biosciences) and incubated for 1 h with 2.0 μ g anti-syntaxin-4 mAb and 20 μ l of Protein G Sepharose at 4°C. Precleared pellets and immunoprecipitates were subjected to acrylamide gel electrophoresis.

Fluorescent antibody cell sorting analysis of granule fractions

Granule fractions exhibiting peak EPO and MPO activities were formalin-fixed, labelled with primary antibodies (5 μ g/ml) and detected by phycoerythrin (PE)-conjugated secondary antibodies (Cedarlane) as described in a previous study (9). Permeabilized eosinophil granules (in 0.1% saponin) and nonpermeabilized neutrophil granules were examined for expression of markers for crystalloid granules (MBP) and azurophilic granules (CD63). The fluorescence intensity was recorded for the entire granule population (ungated) and percentage of positive events determined in respect to isotype control antibodies (percentage gated in M1). Results from three separate experiments were averaged, the SEM calculated, and the significance from isotype controls ($P < 0.05$) were determined using a paired *t*-test.

Cell permeabilization and Ca²⁺ and GTP γ S-induced mediator secretion

Based on a previous report (21), cells were washed (3x) in BSS (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 20 mM PIPES, pH 6.8), resuspended at 1×10^6 cells/ml, added in triplicate to V-well plates (50 000 cells/well), and permeabilized in the presence or absence of isotype control antibodies or SNARE-specific antibodies with 1.0 μ g/ml (eosinophils) or 0.1 μ g/ml (neutrophils) SLO. After 2 min of permeabilization, degranulation was triggered by pCa5 (approximately 10 μ M Ca²⁺) supplemented with 10 μ M GTP γ S and 1 mM ATP for 10 min at 37°C. Cells incubated at pCa7 (approximately 0.1 μ M Ca²⁺) and 1 mM ATP served as negative controls. Reactions were terminated at 10 min with 100- μ l cold BSS, plates centrifuged at 350 *g* for 5 min at 4°C and mediators examined from cell supernatants. To determine the kinetic uptake of exogenously added antibodies, cells were incubated in 1.0 μ g/ml PE-conjugated anti-mouse antibodies (Cedarlane) in the presence of SLO. Cell populations were warmed for 1 min at 37°C to initiate permeabilization and subsequently examined by flow cytometry for changes in fluorescence intensity for an additional 8.5 min.

Eosinophil peroxidase and MPO release were determined using 3,3',5,5'-tetramethylbenzidine (Sigma). The percentage-specific release of EPO/MPO was determined according to the formula: $[(OD_{450nm} \text{ test} - OD_{450nm} \text{ negative control}) / (OD_{450nm} \text{ lysed cells} - OD_{450nm} \text{ negative control})] \times 100$. Enzyme-linked immunosorbent assays were used to determine secreted

matrix metalloproteinase-9 (MMP-9; R&D Systems, Minneapolis, MN) and lactoferrin (LF; Oxis Research, Portland, OR) from neutrophils. Eosinophil-derived neurotoxin (EDN) was determined by radioimmunoassay (22). Statistical significance from isotype controls ($P < 0.05$) was determined by ANOVA, followed by Tukey's multiple comparison test. For graphical representation, the mean percentage release of control from three separate experiments was calculated according to the formula: $[(\text{test Ab} - \text{negative control}) / (\text{isotype Ab} - \text{negative control})] \times 100$ and expressed with error bars representing SEM.

Results

Expression of v-SNAREs in membrane and granule fractions of eosinophils and neutrophils

The expression of v-SNAREs in granulocytes was determined by Western blot analysis of subcellular fractions containing peak marker activities for granules, membranes and cytosol (Fig. 1A,B). Vesicle-associated mem-

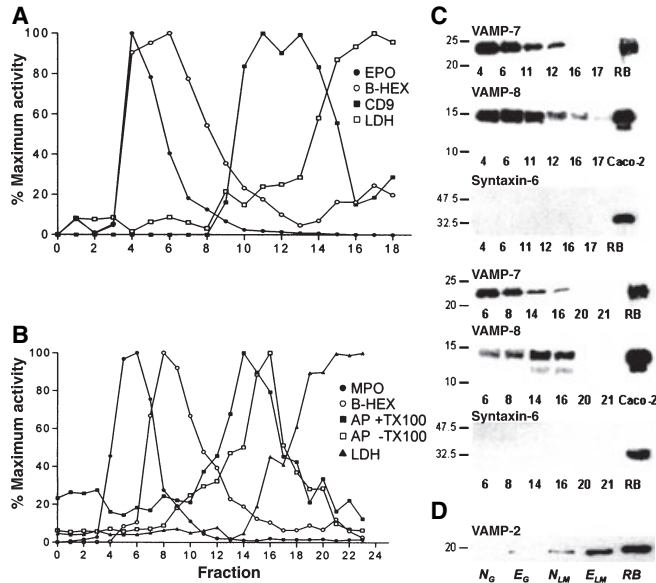


Figure 1. Expression of vesicle-associated membrane protein (VAMP)-7 and VAMP-8 in granule and plasma membrane fractions. Eosinophil (A) and neutrophil (B) subcellular fractions were generated and examined for marker protein activities: eosinophil peroxidase/ β -hexosaminidase (eosinophil granules), myeloperoxidase (neutrophil azurophilic granules); alkaline phosphatase (neutrophil membranes); CD9 (eosinophil membranes); and lactate dehydrogenase (cytosol) as indicated in the *Methods* section. (C) Fractions with peak activity for marker proteins were examined in duplicate by Western blot analysis for expression of VAMP-7, VAMP-8 and syntaxin-6. Fractions (in order of decreasing density) are indicated below each panel and molecular-weight markers on the left. Immunoreactive bands are shown in respect to positive controls: 100 μ g rat brain homogenate (RB) and 100 μ g CaCo-2 cell lysate. (D) Membrane pellets (LM) generated from high-speed centrifugation and 50–100 μ g granule (G) fractions from eosinophils (E) and neutrophils (N) were examined for VAMP-2 immunoreactivity with rat brain (RB) homogenate positive control (50 μ g).

brane proteins-7 and -8 were expressed in fractions containing crystalloid and azurophilic granules, as well as other membrane-bound compartments, in eosinophils and neutrophils respectively (Fig. 1C). In contrast, VAMP-2 was not detectable in subcellular fractions (data not shown) or pooled crystalloid or azurophilic granules (Fig. 1D). Vesicle-associated membrane protein-2 protein was, however, detected following high-speed centrifugation (100 000 g) of 2–3 pooled cell membrane-positive fractions (Fig. 1D). In three separate experiments, syntaxin-6 was not detected in either eosinophil or neutrophil fractions despite consistent immunoreactive bands in rat brain controls (Fig. 1C). Concentrated granule (50–100 μ g) and membrane fractions (25–50 μ g), from neutrophils were similarly found to contain no detectable syntaxin-6 (data not shown).

The localization of VAMP-7 and VAMP-8 to eosinophil and neutrophil granules was confirmed by flow cytometric analysis (Fig. 2). Labelling for MBP (69 $\pm$ 3%) and CD63 (59 $\pm$ 7%) indicated significant enrichment of crystalloid and azurophilic granules in eosinophil and neutrophil low-density fractions, respectively ($P < 0.05$). A significant number of eosinophil granules expressed VAMP-7 on their surfaces (22 $\pm$ 5%) similar to azurophilic granules (20 $\pm$ 4%) ($P < 0.05$). Vesicle-associated membrane protein-8 was also detected on granules from both eosinophils (34 $\pm$ 8%) and neutrophils (37 $\pm$ 6%) ($P < 0.05$). In contrast, crystalloid and azurophilic-enriched fractions labelled with

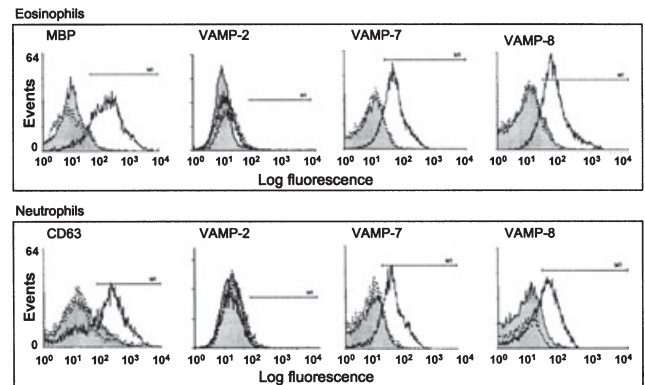


Figure 2. Flow cytometric analysis of vesicle-associated membrane protein (VAMP)-7 and VAMP-8 expression in eosinophil crystalloid and azurophilic-enriched fractions. Nonpermeabilized eosinophil crystalloid granule and neutrophil azurophilic granule fractions were stained with isotype control antibodies [mouse immunoglobulin IgG₁ (IgG₁) or rabbit IgG; grey-filled], or isoform-specific VAMP antibodies (solid black). As positive controls, saponin-permeabilized crystalloid granules were mouse anti-human major basic protein (MBP) monoclonal antibody (mAb) and nonpermeabilized azurophilic granules labelled with mouse anti-human CD63 mAb. Negative controls labelled with secondary antibody alone are shown (dotted black). Figures are representative of three separate experiments.

VAMP-2 mAb exhibited negligible immunofluorescence above isotype controls ($5 \pm 8\%$ and $7 \pm 9\%$ respectively). No significant immunoreactivity for syntaxin-6 was found on granule fractions from either eosinophils or neutrophils (data not shown).

SNARE–SNARE interactions in granulocytes

Immunoprecipitated v/t-SNARE pairings are readily detected in neurons that have vesicles docked to the plasma membrane prior to activation (23). In granulocytes, which do not exhibit docked granules, we anticipated that v/t-SNARE partnering would occur following Ca^{2+} and $\text{GTP}\gamma\text{S}$ -evoked exocytosis. We observed SNAP-23 consistently co-precipitated with syntaxin-4 in granulocytes under both resting (Fig. 3) and activated (data not shown) conditions. Similarly, syntaxin-4 was detected in SNAP-23 immunoprecipitates (data not shown). We were unable to detect immunoreactive bands for either VAMP-2, -7 or -8 in either resting or activated immunoprecipitates (data not shown). The addition of 1 mM *N*-ethylmaleimide, an inhibitor of SNARE complex disassembly, either prior to SLO-permeabilization or following stimulation with Ca^{2+} and $\text{GTP}\gamma\text{S}$ for 10 min, similarly, did not result in v-SNARE co-precipitation with either syntaxin-4 or SNAP-23 (data not shown).

Effects of SNARE-specific antibodies on secretion of granule-derived mediators from permeabilized granulocytes

To assess the rate of antibody diffusion into SLO-permeabilized granulocytes, PE-labelled antibodies were added to permeabilized cells and cells were monitored for increased intracellular fluorescence by flow cytometry. These antibodies rapidly entered cells incubated with

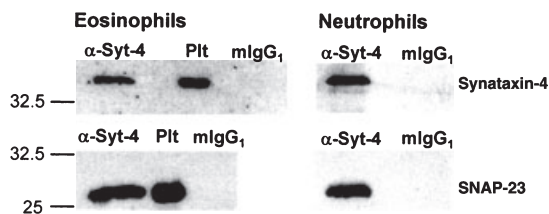


Figure 3. *In vivo* interaction of target-soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs), SNAP-23 and syntaxin-4. Eosinophil and neutrophil light-membrane pellets (20×10^6 cell equivalents; left and right panels respectively) were precleared using control antibodies (mouse IgG₁) and subjected to immunoprecipitation using syntaxin-4 antibodies (α -Syt-4). Western blot analyses of precleared light membranes and syntaxin-4 immunoprecipitates are shown using anti-syntaxin-4 (upper panels) or anti-SNAP-23 (lower panels) antibodies. Immunoreactivity of syntaxin-4 and SNAP-23 was confirmed in respect to concurrently electrophoresed-positive controls, 50 μ g platelet lysate (Plt). Molecular-weight markers are shown on the left.

SLO (within 1 min) and demonstrated increasing immunoreactivity over time (Fig. 4A,B). Titration of SLO revealed a minimum effective dose of 0.1 vs 1.0 μ g/ml SLO for neutrophils and eosinophils, respectively, for rapid antibody entry. These concentrations are within the range of previously reported effective doses, which vary according to cell type (21, 24).

The secretion of EPO and MPO from permeabilized eosinophils and neutrophils, respectively, was triggered by Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ (10 μ M) addition at the time of SLO permeabilization. Longer incubations of 30 and 60 min did not enhance the amount of secreted EPO and MPO (data not shown). Cells stimulated with Ca^{2+} and $\text{GTP}\gamma\text{S}$ released $58 \pm 14\%$ of total EPO, and $68 \pm 8\%$ MPO, which was comparable with cells preincubated with relevant isotype antibodies (mouse IgG₁ and rabbit IgG) released $52 \pm 9\%$ EPO and $70 \pm 9\%$ MPO ($n = 5$). These values were designated as 100% isotype control secretion for SNARE inhibition experiments and mediator release expressed as a percentage of control. To determine the role of different SNAREs in exocytosis, we quantitated secreted granule proteins from SLO-permeabilized cells incubated in the presence of isoform-specific

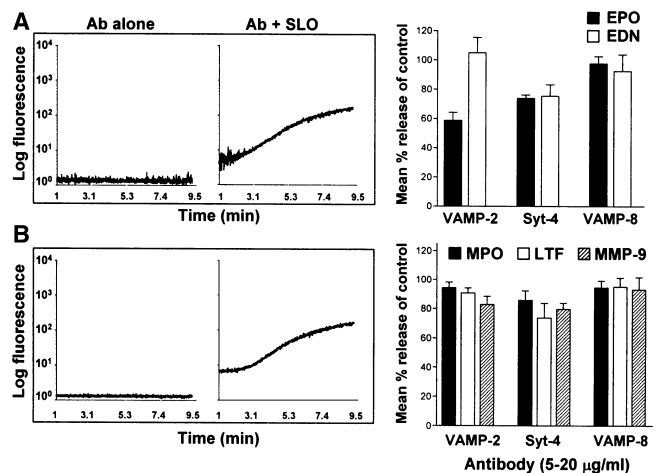


Figure 4. Effects of vesicle-associated membrane protein (VAMP)-2, -8 and syntaxin-4-specific antibodies on granule-derived mediator secretion from permeabilized granulocytes. Eosinophils (A, left) and neutrophils (B, left) were incubated with 1.0 μ g/ml phycoerythrin-secondary antibodies in the absence or presence of 1.0 or 0.1 μ g/ml streptolysin-O (SLO), respectively, for 1 min at 37°C . Cells were then examined continuously by flow cytometry for the change in log-fluorescence intensity for approximately 8.5 min. Human eosinophils (A, right) and neutrophils (B, right) were permeabilized in SLO and incubated in the presence of 5–20 μ g/ml isotype control antibodies (mlgG₁), VAMP-2 mAb, syntaxin-4 mAb (Syt-4) or VAMP-8 rabbit IgG prior to activation with pCa5 (approximately 10 μ M Ca^{2+}) and 10 μ M $\text{GTP}\gamma\text{S}$ for 10 min. Cell supernatants were examined for granule-derived mediators as indicated in *Methods*. Data are expressed as the average percentage of isotype control $\pm$ SEM from three separate experiments. (* $P < 0.05$ from isotype control antibody).

SNARE antibodies. In human eosinophils, VAMP-2 mAb impaired the secretion of EPO ($58 \pm 6\%$) at an effective dose of $5 \mu\text{g/ml}$, but was not potentiated at higher doses (Fig. 4A). In contrast, secretion of EDN was unaffected by VAMP-2 mAb treatment. In neutrophils, VAMP-2 mAb did not significantly inhibit the secretion of MPO or LF. The release of MMP-9 appeared to be slightly more sensitive to VAMP-2 mAb, however, the level of inhibition observed was not statistically different from control (mIgG₁) ($P = 0.058$; Fig. 4B). We were unable to detect any inhibitory effect by VAMP-8 Ab for any of the tested mediators in either eosinophils or neutrophils (Fig. 4A,B).

In contrast, VAMP-7 mAb impaired secretion of all granulocyte-derived mediators in a dose-dependent manner (Fig. 5). In eosinophils, the secretion of EPO and EDN was significantly inhibited in response to $1 \mu\text{g/ml}$ VAMP-7 mAb (Fig. 5A). Similar results were obtained using VAMP-7 mAb for neutrophils, which impaired the secretion of MPO, although slightly higher doses of VAMP-7 mAb were required to inhibit MMP-9 and LF secretion (Fig. 5B).

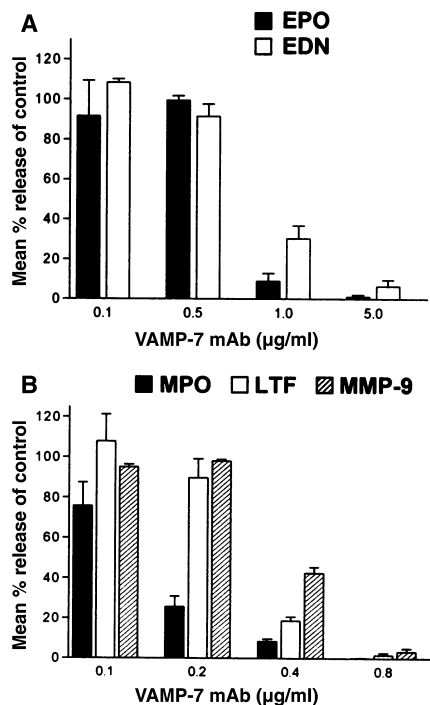


Figure 5. Effect of vesicle-associated membrane protein-7 (VAMP-7) antibody on granule-derived mediator secretion from permeabilized granulocytes. Streptolysin-O-permeabilized eosinophils (A) and neutrophils (B) were incubated in the presence of increasing doses of VAMP-7 mAb or matched concentrations of isotype control antibody (mIgG₁), activated with Ca^{2+} and GTPγS as described in the legend for Fig. 4. Data are expressed as the average percentage of isotype control $\pm$ SEM from three separate experiments. (* $P < 0.05$ from isotype control antibody).

To further address the role of VAMP isoforms, we attempted to examine the effects of Botulinum B-light chain (BoNT-B-LC) on degranulation from permeabilized granulocytes. BoNT-B-LC cleaves VAMP-2 but not VAMP-7 or -8 (25, 26). Our experiments indicated that 10 nM BoNT-B-LC did not significantly impair release of either EDN or MMP-9 from eosinophils and neutrophils, respectively (data not shown). We were unable to measure EPO and MPO release as boiled inactivated toxin (at doses of $>5 \text{ nM}$) interfered with peroxidase measurements, probably due to added dithiothreitol for maintaining LC-subunit activity. We also found that sodium azide ($>0.004\%$) in antibody preparations impaired peroxidase readings from cell lysates. All antibodies utilized in this study were, consequently, azide free.

We sought to determine whether targeted inhibition of t-SNAREs would cause a similar inhibitory response of secreted granule mediator release to VAMP(s). Anti-syntaxin-4 modestly inhibited secretion of EPO and EDN from permeabilized eosinophils (Fig. 4A). A similar small suppressive effect of anti-syntaxin-4 on MMP-9 and LF release was observed in permeabilized neutrophils. Although the release of MPO appeared to be slightly downregulated, the values were not significantly different from controls between the three separate experiments ($P = 0.053$; Fig. 4B). We also examined the effect of targeting the t-SNARE-binding partner of syntaxin-4, SNAP-23. At a dose of $20 \mu\text{g/ml}$, SNAP-23 antibodies yielded values comparable ($P > 0.05$) with syntaxin-4 mAb treatment (data not shown). Finally, we determined that $20 \mu\text{g/ml}$ syntaxin-6 mAb has negligible effect on released mediators (data not shown), which was consistent with our finding that detectable syntaxin-6 protein was not found in these cells (Fig. 1C).

Discussion

The SNARE isoforms localized to eosinophil crystalloid granules and neutrophil azurophilic granules have not been previously characterized. We have shown the v-SNAREs, VAMP-7 and -8, but not VAMP-2, are localized to these granule compartments. Antibody-mediated inhibition of VAMP-7, but not VAMP-8, impaired the release of crystalloid granule mediators, EPO and EDN, and the azurophilic granule-specific mediator, MPO, in a dose-dependent manner. Our observations are consistent with previous studies that have shown VAMP-7 is localized to CD63⁺ lysosomes, which are similar to CD63⁺ crystalloid and azurophilic granules (14, 27, 28). In human neutrophils, we also found VAMP-7 mAb dose-dependently impaired the release of mediators from secondary (LF) and tertiary granules (MMP-9). These findings indicate that VAMP-7 and VAMP-8 expression was not restricted to distinct granule populations, but were also detected in low-

density membrane-enriched fractions. Other studies have similarly supported that these v-SNAREs are not strictly compartment-specific. For example, VAMP-7 has been implicated in exocytosis of numerous granule populations in secretory cells (17, 25, 27) and VAMP-8 in either endosome or granule trafficking (15, 16). Our data indicate that exocytosis of separate granule populations cannot be attributed to differences in SNARE isoform expression alone. This finding is consistent with previous studies that have shown SNAREs can interact with multiple partners and may perform more than one trafficking role *in vivo* (29).

In contrast to VAMP-7 mAb, we found VAMP-8-specific antibodies did not demonstrate any inhibitory effect on the release of any of the examined eosinophil or neutrophil mediators. Both antibodies used in the current study recognize the cytoplasmic NH₂-terminal region required for SNARE-SNARE interactions, and specifically label native proteins (13, 18) which was confirmed in this study by flow cytometry. Our observations suggest that VAMP-8 is not critical for mediator release from human granulocytes. However, our data do not conclusively rule out a functional role for VAMP-8. To date, the only ascribed function of SNAREs is to catalyse membrane fusion. It is possible that VAMP-8 may play a role in specific patterns of mediator release, such as granule-granule fusion during compound exocytosis. Alternatively, the expression of VAMP-8 on the surface of granules could be indicative of a role in the fusion of granule with either endosomes or small vesicles.

In both eosinophils and neutrophils, secretory vesicles are rapidly mobilized and exocytosed following agonist-induced cell activation as a first line of defence (4, 5). In eosinophils, secretory vesicles have also been postulated to account for the release of mediators from cytoplasmic crystalloid granules, which exhibit evidence of content loss by microscopic analysis (termed piecemeal degranulation) (7, 30). It has been postulated that membrane budding of crystalloid granules may be a potential mechanism for piecemeal degranulation. Alternatively, secretory vesicles could comprise a separate population that function as transport vesicles for the release of crystalloid granule cargo (3, 4). In this study and in a previous report (9), we have shown that VAMP-2 is localized to secretory vesicles, but is not detected on large crystalloid granules. This observation suggests that at least a proportion of the secretory vesicle pool comprises a separate population that is not derived from crystalloid granules.

In human eosinophils, VAMP-2 vesicles were co-localized with RANTES and implicated in vesicle-mediated release of this cytokine following treatment with interferon- γ (9). In the present study, we have provided evidence that VAMP-2 is functionally implicated in exocytosis of EPO, but not EDN (2). Both of these mediators are predominantly localized to crystalloid granules, but EPO has also been reported in secretory

vesicles by electron microscopy (3). However, the inhibitory effect of VAMP-2 mAb was not as pronounced as that of VAMP-7 mAb for EPO. This suggests that, under our experimental conditions, the bulk of secreted EPO and EDN released by crystalloid granule fusion is dependent on VAMP-7, but not on VAMP-2. A study by Hoffmann et al. (31) similarly demonstrated that VAMP-2 was expressed in secretory vesicles, but not crystalloid granules, by subcellular fractionation of human eosinophils. They reported that tetanus toxin (TeNT) impaired the release of ECP from SLO-permeabilized eosinophils, although this was not compared with an inactivated toxin control. We attempted to confirm our findings with VAMP-2 mAb by examining BoNT-B-LC (which cleaves VAMP-2 at the same site as TeNT). Although reagent incompatibility prevented our analysis of EPO, we found no inhibitory effect of BoNT-B-LC on EDN release. Unlike the assays for MPO and EPO, the assay for EDN was not sensitive to BoNT-B-LC-related reagents. Our observations suggest that VAMP-2 is predominantly involved in exocytosis of a preformed small secretory vesicle pool that is distinct from crystalloid granules.

Previous studies have provided evidence that VAMP-2 is localized to multiple compartments in neutrophils, including small vesicles, secondary granules and tertiary granules (10, 32). Mollinedo et al. (10) showed that VAMP-2 mAb impaired the surface upregulation of the common secondary/tertiary granule marker, CD66b, but not the azurophilic granule marker, CD63, in electroporated neutrophils. Consistent with this study, we observed that the same VAMP-2 mAb had a negligible effect on release of the specific azurophilic granule mediator, MPO. However, we also found that VAMP-2 did not significantly impair exocytosis of LF or MMP-9 from neutrophils. This discrepancy may be due to differences in experimental methods because we have evaluated secreted mediators from secondary and tertiary granules, rather than upregulation of surface markers. Our observation that VAMP-2 is detected only in concentrated high-speed membrane pellets from human neutrophils is consistent with previous study by Brumell et al. (32) that suggested a significant amount of this v-SNARE was localized to small secretory vesicles that pellet only at high-centrifugation speeds.

The t-SNAREs, SNAP-23 and syntaxin-4 are each capable of interacting with VAMP-2, -7 and -8 *in vitro* (8, 13, 33) which suggests that they have the potential to support exocytosis of multiple granule compartments. SNAP-23 and syntaxin-4 are localized to the plasma membrane in eosinophils (11), neutrophils (10, 12), but are also detected on other intracellular organelles. We detected a t-SNARE complex of SNAP-23 and syntaxin-4 in membrane fractions of resting and Ca²⁺ and GTP γ S-activated granulocytes, but we were unable to find immunoreactivity for v-SNAREs (VAMP-2, -7 and -8) in syntaxin-4 immunoprecipitates (with or without

N-ethylmaleimide). It is possible that rapid recycling of SNARE complexes limited the ability to detect v/t-SNARE pairings by immunoprecipitation. For example, in neurons it has been shown that v/t-SNARE complexes are rapidly disassembled and VAMPs recycled within seconds following mediator release (34). Alternatively, it is also possible that v/t-SNARE interactions may have been obscured by additional regulatory molecules that are recruited to the granule-docking site for assembly of SNARE complexes and granule-membrane fusion. We anticipate that such epitope obstruction may have limited the inhibitory effect of SNAP-23 and syntaxin-4 antibodies on mediator release. The inhibitory effect was moderate for both the t-SNARE antibodies, whereas VAMP-7 and VAMP-2 mAbs (for EPO) were more effective inhibitors of secretion in permeabilized cells. In contrast to a previous report, which indicated a role for syntaxin-6 in neutrophil secretion (12), we were unable to detect this t-SNARE in either subcellular fractions or high-speed membrane pellets from eosinophils and neutrophils. We also confirmed that doses up to 20 µg/ml of syntaxin-6 mAb did not exert any inhibitory effect on the secretion of granule mediators from either cell type.

In summary, we have identified VAMP-7 as a predominant SNARE involved in granule-derived mediator release from eosinophils and neutrophils. However, this conclusion is limited by the model of secretion used in this study. In support of our findings, other studies suggest VAMP-7 and/or VAMP-8 are implicated in secretion from haematopoietic cell types. Both VAMP-7 and VAMP-8 were previously localized to granules of rat

basophilic cells (RBL-2H3), although a functional role for either isoform in exocytosis has not yet been clearly demonstrated (13). In human platelets, the addition of a recombinant cytoplasmic domain of VAMP-8 was shown to selectively impair the release of dense-granule mediators from permeabilized cells (16). Finally, VAMP-7 was recently implicated in exocytosis of late endocytic vesicles from RAW.264 macrophages (35). Taken together, these findings suggest that a conserved family of fusion proteins may regulate the secretion of inflammatory mediators from different cell types. Further studies are required to determine the signalling molecules involved in the recruitment of granule populations to the plasma membrane and their role in SNARE assembly. These studies are necessary in the pursuit of candidate proteins that may prove to be selective targets for the modulation of secretory function of inflammatory cells.

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References

- Adamko DJ, Odemuyiwa SO, Vethanayagam D, Moqbel R. The rise of the phoenix: the expanding role of the eosinophil in health and disease. *Allergy* 2005;**60**:13–22.
- Lacy P. The role of rho GTPases and SNAREs in mediator release from granulocytes. *Pharmacol Ther* 2005;**107**:358–376.
- Dvorak AM, Ackerman SJ, Furitsu T, Estrella P, Letourneau L, Ishizaka T. Mature eosinophils stimulated to develop in human-cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation. *Am J Pathol* 1992;**140**:795–807.
- Moqbel R, Lacy P. Exocytotic events in eosinophils and mast cells. *Clin Exp Allergy* 1999;**29**:1017–1022.
- Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 2003;**5**:1317–1327.
- Fasshauer D, Sutton RB, Brunger AT, Jahn R. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA* 1998;**95**:15781–15786.
- Logan MR, Odemuyiwa SO, Moqbel R. Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. *J Allergy Clin Immunol* 2003;**111**:923–932.
- Fasshauer D, Antonin W, Margittai M, Pabst S, Jahn R. Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. *J Biol Chem* 1999;**274**:15440–15446.
- Lacy P, Logan MR, Bablitz B, Moqbel R. Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals. *J Allergy Clin Immunol* 2001;**107**:671–678.
- Mollinedo F, Martin-Martin B, Calafat J, Nabokina SM, Lazo PA. Role of vesicle-associated membrane protein-2, through Q-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor/R-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor interaction, in the exocytosis of specific and tertiary granules of human neutrophils. *J Immunol* 2003;**170**:1034–1042.
- Logan MR, Lacy P, Bablitz B, Moqbel R. Expression of eosinophil target SNAREs as potential cognate receptors for vesicle-associated membrane protein-2 in exocytosis. *J Allergy Clin Immunol* 2002;**109**:299–306.

12. Martin-Martin B, Nabokina SM, Blasi J, Lazo PA, Mollinedo F. Involvement of SNAP-23 and syntaxin 6 in human neutrophil exocytosis. *Blood* 2000;**96**:2574–2583.
13. Paumet F, Le MJ, Martin S, Galli T, David B, Blank U et al. 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. *J Immunol* 2000;**164**:5850–5857.
14. Advani RJ, Yang B, Prekeris R, Lee KC, Klumperman J, Scheller RH. VAMP-7 mediates vesicular transport from endosomes to lysosomes. *J Cell Biol* 1999;**146**:765–776.
15. Antonin W, Holroyd C, Tikkanen R, Honing S, Jahn R. The R-SNARE endobrevin/VAMP-8 mediates homotypic fusion of early endosomes and late endosomes. *Mol Biol Cell* 2000;**11**:3289–3298.
16. Polgar J, Chung SH, Reed GL. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. *Blood* 2002;**100**:1081–1083.
17. Martinez-Arca S, Coco S, Mainguy G, Schenk U, Alberts P, Bouille P et al. A common exocytotic mechanism mediates axonal and dendritic outgrowth. *J Neurosci* 2001;**21**:3830–3838.
18. Muzerelle A, Alberts P, Martinez-Arca S, Jeannequin O, Lafaye P, Mazie JC et al. Tetanus neurotoxin-insensitive vesicle-associated membrane protein localizes to a presynaptic membrane compartment in selected terminal subsets of the rat brain. *Neuroscience* 2003;**122**:59–75.
19. Moqbel R, Barkans J, Bradley BL, Durham SR, Kay AB. Application of monoclonal antibodies against major basic protein (BMK-13) and eosinophil cationic protein (EG1 and EG2) for quantifying eosinophils in bronchial biopsies from atopic asthma. *Clin Exp Allergy* 1992;**22**:265–273.
20. Lacy P, Abdel-Latif D, Steward M, Musat-Marcu S, Man SF, Moqbel R. Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects. *J Immunol* 2003;**170**:2670–2679.
21. Larbi KY, Gomperts BD. Practical considerations regarding the use of streptolysin-O as a permeabilising agent for cells in the investigation of exocytosis. *Biosci Rep* 1996;**16**:11–21.
22. Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989;**142**:2393–2400.
23. Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH. SNARE complex formation is triggered by Ca²⁺ and drives membrane fusion. *Cell* 1999;**97**:165–174.
24. Rosales JL, Ernst JD. GTP-dependent permeabilized neutrophil secretion requires a freely diffusible cytosolic protein. *J Cell Biochem* 2000;**80**:37–45.
25. Galli T, Zahraoui A, Vaidyanathan VV, Raposo G, Tian JM, Karin M et al. A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. *Mol Biol Cell* 1998;**9**:1437–1448.
26. Humeau Y, Doussau F, Grant NJ, Poulain B. How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie* 2000;**82**:427–446.
27. Coco S, Raposo G, Martinez S, Fontaine JJ, Takamori S, Zahraoui A et al. Subcellular localization of tetanus neurotoxin-insensitive vesicle-associated membrane protein (VAMP)/VAMP7 in neuronal cells: evidence for a novel membrane compartment. *J Neurosci* 1999;**19**:9803–9812.
28. Rao SK, Huynh C, Proux-Gillardeaux V, Galli T, Andrews NW. Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. *J Biol Chem* 2004;**279**:20471–20479.
29. Tsui MM, Tai WC, Banfield DK. Selective formation of Sed5p-containing SNARE complexes is mediated by combinatorial binding interactions. *Mol Biol Cell* 2001;**12**:521–538.
30. Lacy P, Mahmudi-Azer S, Bablitz B, Hagen SC, Velazquez JR, Man SF et al. Rapid mobilization of intracellularly stored RANTES in response to interferon-gamma in human eosinophils. *Blood* 1999;**94**:23–32.
31. Hoffmann HJ, Bjerke T, Karawajczyk M, Dahl R, Knepper MA, Nielsen S. SNARE proteins are critical for regulated exocytosis of ECP from human eosinophils. *Biochem Biophys Res Commun* 2001;**280**:172–176.
32. Brumell JH, Volchuk A, Sengelov H, Borregaard N, Cieutat AM, Bainton DF et al. Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. *J Immunol* 1995;**155**:5750–5759.
33. Martinez-Arca S, Rudge R, Vacca M, Raposo G, Camonis J, Proux-Gillardeaux V et al. A dual mechanism controlling the localization and function of exocytic v-SNAREs. *Proc Natl Acad Sci USA* 2003;**100**:9011–9016.
34. Sankaranarayanan S, Ryan TA. Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. *Nat Cell Biol* 2000;**2**:197–204.
35. Braun V, Fraissier V, Raposo G, Hurbain I, Sibarita JB, Chavrier P et al. TI-VAMP/VAMP7 is required for optimal phagocytosis of opsonised particles in macrophages. *EMBO J* 2004;**23**:4166–4176.