

# Small-Interfering RNA-Mediated Identification and Regulation of the Ternary SNARE Complex Mediating RBL-2H3 Mast Cell Degranulation

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## Abstract

Dysregulation of mast cell function contributes to allergic and autoimmune disease that affects more than 70 million persons in the United States alone. Identifying novel mast cell targets that mediate disease or disease progression is required for the development of innovative therapeutics for the treatment of allergy/asthma and autoimmune disease. RNA interference technologies offer hope both as basic research tools for target identification and as potential, novel, specific therapeutics. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are a family of evolutionarily conserved proteins that have been postulated to mediate the transport and fusion of inflammatory mediator-laden vesicles to the membrane in mast cells leading to their subsequent exocytosis. The functional role(s) of specific SNARE family member complexes in mast cell degranulation has not been fully elucidated. Here, we characterize the functional importance of SNARE complexes in FcεRI receptor-mediated degranulation of RBL-2H3 cells utilizing RNA interference. We demonstrate that ternary SNARE complexes of synaptosomal-associated protein-23, Syntaxin 4 and vesicle-associated membrane protein-7 (VAMP-7) or VAMP-8 are directly involved in mast cell degranulation. Additionally, we evaluate the siRNAs directed against these molecules as potential therapeutic agents for disease intervention. These studies have identified specific SNARE proteins and complexes that serve as novel targets for the development of siRNA therapies to treat allergic and autoimmune disease.

## Introduction

The activation of mast cell degranulation pathways has been implicated as an important mediator of allergic disease and more recently as an initiator and contributor to autoimmune disease [1]. Mast cells are phagocytic granulocytes derived from myeloid progenitors in bone marrow compartments that play a critical role in innate immunity. As vital sentinel cells, they combat invading microorganisms at the tissue-environment interfaces located in the gut, skin, lungs and nasal passages [1]. Mast cells are also key regulators of the adaptive immune system promoting antigen presentation, differentiation of naive T cells into helper T cells and the induction of acquired immunity towards parasites via IgE [2–4]. Mast cells are also key players in maintaining immunological tolerance by interacting with regulatory T cells.

Dysregulation of mast cell function leads to allergic and autoimmune disease and contributes to heart disease and tumour progression. Mast cells contribute to immune responses by the release of inflammatory mediators through a process known as regulated exocytosis. These mediators include histamine, heparin, serine proteases and specific cytokines. Leukotrienes, prostaglandins, thromboxanes and additional cytokines are released during later-phase reactions. Activation of mast cells occurs via FcεRI engagement by IgE crosslinked with antigen and through direct interaction with microorganisms mediated by conserved host toll-like receptors.

A hallmark of regulated exocytosis is the storage of intracellular pools of inflammatory mediators, hormones or neurotransmitters in preformed granules/vesicles (which are released via a fusion mechanism with the plasma membrane). This mechanism is activated through

receptor stimulation or by membrane depolarization via second messengers, e.g.  $\text{Ca}^{2+}$  [5]. Vesicle transport and fusion to the membrane and subsequent exocytosis is mediated by a family of proteins known as the SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) [5–9]. The SNAREs are evolutionarily conserved proteins found in all eukaryotic cells. They are composed of an  $\alpha$ -helix domain structure highlighted by a SNARE motif, which is a stretch of 60–70 amino acids arranged in a heptad repeat [5–9]. Different sets of SNAREs on opposing membranes associate into stable core complexes formed by four intertwined parallel  $\alpha$ -helices contributed by individual SNARE motifs [6]. After fusion, SNAREs are recycled via dissociation, mediated by the ATP-dependent co-factor *N*-ethylmaleimide-sensitive factor (NSF) and  $\alpha$ -soluble NSF attachment protein ( $\alpha$ SNAP). Models predict that SNAREs drive opposing membranes together by exerting mechanical forces on the membranes, thus initiating fusion once phospholipid bilayers come into close proximity. Bundles of SNARE proteins bridging opposing membranes lead to the exclusion of water allowing lipid mixing and subsequent membrane fusion.

The  $\text{Ca}^{2+}$ -regulated SNARE proteins are a subset of the SNARE family that share a multistep membrane fusion characteristic. This subset of SNAREs is utilized in regulated exocytosis where vesicles must first dock at a target membrane and then only release the vesicular cargo when signalled by the cell. Members of the  $\text{Ca}^{2+}$ -regulated SNARE subfamily include the neuronal SNAREs, responsible for calcium-triggered neurotransmitter release in neurons [6, 10]. Herein, we identify SNARE proteins and the complexes they form when driving mast cell exocytosis (degranulation). The identification of small-molecule modulators that specifically regulate mast cell degranulation is an important step towards the identification of novel therapies for the treatment of allergy, asthma and autoimmune disease. To that end, our siRNA method is a 'proof of concept' step towards a novel therapeutic strategy.

It is not clearly evident from the literature which synaptosomal-associated protein (SNAP), vesicle-associated membrane protein (VAMP) and Syntaxin family members are the functional SNAREs expressed on mast cell granule membranes and the plasma membranes as multiple contradictory reports have implicated several members of each family including SNAP-23, -25, Syntaxin 2, 3 and 4 and VAMP-1, -2, -3, -7 and -8 [11–16]. The ternary SNARE complex mediating mast cell degranulation has not been functionally characterized.

Several groups have attempted to functionally characterize SNARE proteins in mast cells by permeabilizing cells with streptolysin-O and using recombinant SNARE proteins or antibodies against SNARE proteins to inhibit degranulation [13, 17, 18]. However, the use of this

toxin to 'poke' holes in live cells and then introducing macromolecules into the cell through these large pores to dissect function is not physiologically relevant. Several immunoprecipitation studies aimed at characterizing the ternary SNARE complex in mast cells have yielded results that have not been reproducible in other studies resulting in as many as three different mast cell ternary complex models [12, 14, 16, 19–22]. These data have led to uncertainty over the physiologically relevant functional ternary complexes that mediate mast cell degranulation.

Over the past 10 years, RNA interference has had a deep impact as a basic research tool for the study of gene and protein function. Here, we take advantage of siRNA as a basic research tool while simultaneously evaluating the initial characteristics of these molecules as therapeutic agents for disease intervention [23]. We have developed a transfection method allowing us to utilize RNAi technology to selectively target and downregulate SNARE function in mast cell degranulation assays. We identify distinct functional ternary complexes composed of SNAP-23, Syntaxin 4 and either VAMP-7 or VAMP-8 in activated RBL-2H3 mast cells that mediate degranulation.

## Materials and methods

**Cell lines/antibodies.** RBL-2H3 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in minimum essential media containing Earle's salts, L-glutamine and high glucose supplemented with 15% FCS (Invitrogen, Carlsbad, CA, USA) and Normocin (50 mg/500 ml media) (Amaxa, Gaithersburg, MD, USA). Anti-VAMP-2 antibody (Cl69.1) and anti-Syntaxin 1a (78.3) monoclonal antibodies were purchased from Synaptic Systems (Göttingen, Germany). Anti-SNAP-23 (S2194), anti-SNAP-25 (S9684), anti-VAMP-2 (V1389), anti-VAMP-8 (V7514) and anti-Actin (A2066) polyclonal serum were purchased from Sigma (St. Louis, MO, USA). Anti-SNAP-25 (SP12) monoclonal antibody was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Anti-syntaxin 4 polyclonal serum was a kind gift of Dr. Ulrich Blank (INSERM, Paris, France). Anti-syntaxin (C19) and anti-syntaxin 3 (N17) polyclonal serum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). VAMP-7 (ab56188) polyclonal serum was purchased from Abcam (Cambridge, MA, USA).

**Electrophoresis/immunoblotting.** RBL-2H3 lysates were prepared by lysing cells at  $1\text{e}^6$ – $2.5\text{e}^7$  cells/ml in triton X-100 lysis buffer (1% triton X-100, 1X protease inhibitor cocktail, 10 mM TRIS (pH 8.0) and 150 mM NaCl) for 1 h on ice and cleared by centrifugation at 20,000 g for 20 min at 4 °C. Alternatively, cells were lysed in denaturing lysis buffer (1% SDS, 50 mM TRIS (pH 7.5), 5 mM EDTA, 10 mM DTT, 1X protease inhibitor

cocktail and 15 U/ml DNase I) for 5 min at 95 °C, diluted 1:10 with triton X-100 lysis buffer and passed through a 25-G needle to shear DNA. Lysate was then cooled to 4 °C and cleared as described above. For activation of RBL-2H3, cells were incubated with 1 µg/ml ionomycin (Sigma) for 5 min at 37 °C in culture media and lysed as described above. Samples for electrophoresis were diluted with 2X SDS sample buffer, ±10X reducing agent (Invitrogen) or 0.2 M DTT, boiled for 5 min, and 25 µl of lysate was electrophoresed on 10–20% tris-glycine gels (Invitrogen) for 1.5 h at 150 V. Control rat brain lysates (5 µg/lane; Sigma), PC12, JAR and MM142 cell lysates (5–25 µg/lane; Santa Cruz Biotechnology, Inc.) were sometimes run as controls. Gels were then transferred to nitrocellulose in 1X transfer buffer (Invitrogen) plus 10% methanol for 2 h at 30 V. Membranes were blocked overnight at 4 °C in blotting buffer (2% BSA/TBST, 10 mM TRIS pH 8.0, 150 mM NaCl and 0.5% TWEEN-20). Primary antibodies were incubated in blotting buffer at manufacturer-recommended dilutions for 2 h at room temperature and washed three times with TBST. Horseradish peroxidase-conjugated secondary antibodies were incubated with blots after washing at a 1:2000 dilution in blotting buffer, washed three times in TBST and visualized on an Alpha Innotech (San Leandro, CA, USA) FluorChem SP phospho-imager after incubation for 5 min with chemiluminescent substrate solution (Thermo Scientific, Rockford, IL, USA). Relative protein band intensities were determined by densitometry of non-saturated blots utilizing Alpha Innotech Alpha-EaseFC software calculating integrated density values (IDV) and comparing sample bands to actin control bands and calculating relative expression using the following formula:

Relative intensity of sample band = IDV sample/IDV actin

Calculation of percent control: (siRNA-treated relative intensity/control siRNA relative intensity) \*100

**Immunoprecipitation.** SNAP-23 and Syntaxin 4 polyclonal IgG were covalently linked to protein G agarose (Sigma) [24]. Lysates of activated, *N*-ethylmaleimide (NEM)-treated RBL-2H3 cells were precleared with 20 µl of protein G agarose slurry for 1 h at 4 °C. After centrifugation, 20 µl of antibody-protein G slurry was added to the lysates and incubated overnight at 4 °C on a rotating shaker. Upon centrifugation, agarose was washed four times with IP buffer followed by a wash in PBS, and antigen was eluted in 2X SDS buffer by boiling for 5 min. Proteins were then electrophoresed as described earlier, and SNAREs were detected via Western blot.

**In vitro degranulation assay.** Degranulation assays were modified from published protocols [25, 26]. Briefly,  $1.5 \times 10^5$ – $2.5 \times 10^5$  RBL-2H3 cells were plated overnight in 500 µl of culture media in 24-well tissue culture plates

in the presence or absence of 0.3 µg/ml anti-DNP IgE (Sigma) to sensitize cells for antigen-induced degranulation. Cells were then washed 1X in 500 µl HEPES-buffered saline (140 mM NaCl, 5 mM KCL, 1 mM CaCl<sub>2</sub>, 0.6 mM MgCl<sub>2</sub>, 0.1% glucose, 0.1% BSA and 10 mM HEPES) and re-suspended in 300 µl HEPES-buffered saline in the presence of 100 ng/ml BSA-DNP (Molecular Probes, Eugene, OR, USA) for antigen-induced degranulation, or 1 µg/ml ionomycin for ionomycin-induced degranulation. Degranulation was carried out at 37 °C for 1 h and was quantitated by measurement of β-hexosaminidase activity via a colorimetric biochemical assay. After incubation, supernatant was removed, and cells were lysed in 500 µl of 0.2% triton X-100 in HEPES-buffered saline for 2 min at room temperature (for calculation of total β-hexosaminidase activity). Fifty microlitres of supernatant and 50 µl of lysate were incubated in separate wells of a 96-well microtitre plate with an equal volume of substrate (4 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) diluted in low pH citrate buffer (49.5% 0.05 M citric acid/50.5% 0.05 M trisodium citrate). After 1 h incubation at 37 °C, the reaction was terminated by the addition of pH 10 buffer (150 µl/well of a 1:1 ratio of 167 mM Na<sub>2</sub>CO<sub>3</sub> and 167 mM NaHCO<sub>3</sub>). Activity was then visualized by measuring absorbance at 405 nm on a spectrophotometer. Per-cent degranulation was then determined using the following formula:

$$\% \text{ degranulation} = ((A_{405\text{nm}} \text{ supernatant}) / (A_{405\text{nm}} \text{ lysate} + A_{405\text{nm}} \text{ supernatant})) * 100$$

Degranulation data are presented as % control with control siRNA set to 100%.

**siRNA transfections.** Amaxa nucleofection: RBL-2H3 cells were resuspended at  $1 \times 10^6$  cells/100 µl in Amaxa nucleofection solution L (Amaxa) [26] and transferred to electroporation cuvettes, and siRNA was added at various concentrations, and cells were electroporated using Amaxa program L-029. Five hundred microlitres of prewarmed culture media was added to each cuvette, and cells were then added to 1.5-ml prewarmed culture media in 6-well plates. Cells were incubated for 24–48 h, and cells were assessed for transfection efficiency [using 100 nM siGLO siRNA or 2 µg of a green fluorescent protein (GFP)-expressing plasmid; pmaxGFP (Amaxa)] by fluorescence microscopy or by using flow cytometry on FACScan (BD Biosciences, Franklin Lakes, NJ, USA) using a FITC filter. For siRNA knock-down experiments, cells were incubated for 24–48 h and then washed and lysed for RNA isolation or SDS-PAGE/immunoblotting or transferred to 24-well plates for degranulation assays.

**RNA isolation/reverse transcription/real-time. PCR** Total RNA was isolated from RBL-2H3 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and TRIZOL

reagent (Qiagen) according to the manufacturer's instructions. Briefly, media from 6-well plates were removed and cells washed one time with PBS. Cells were then homogenized by the addition of 1 ml TRIZOL. Two hundred millilitres of chloroform was added to the TRIZOL lysate, mixed and incubated for 3 min at room temperature. The solution was then centrifuged at 15,000 *g* in a microcentrifuge for 10 min at 4 °C. Following centrifugation, the aqueous phase was transferred to a new tube, and one volume of 70% ethanol was added to the tube, mixed and transferred to the RNeasy spin column and purification continued as described in the manufacturer's instructions. RNA was quantitated via spectrophotometry at 260 nm and quality determined by 260/280 ratios.

Reverse transcription was carried out using the Taqman Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA, USA) using 1 µg of total RNA. Cycle times were as follows: 25 °C 10 min, 48 °C 40 min, 95 °C 5 min run on an Applied Biosystems GenAmp PCR system 9700. Quantitative PCR (qPCR) was performed using PCR mastermix (Applied Biosystems) and 2–4 µl of cDNA using the following cycles: 95 °C 20 s 1 cycle, 95 °C 1 s–60 °C 20 s 40 cycles in the presence of 1X FAM (carboxyfluorescein) dye-labelled rat GAPDH (99999916\_s1), rat  $\beta$  actin (00667869\_m1), rat SNAP-23 (01514334\_m1), rat VAMP-8 (00582868\_m1), rat VAMP-7 (00585478\_m1), rat VAMP-2 (01465442\_m1), rat Syntaxin 4 (00579648\_m1), rat Syntaxin 3 (00579637\_m1) and rat Syntaxin 2 (00709358\_m1) Taqman gene expression assays sets (Applied Biosystems) using the 7900 HT fast real-time PCR system (Applied Biosystems).

**Calculation of mRNA knock-down.** mRNA knock-down was calculated via qPCR utilizing the ddCt analysis (delta–delta cycle time, [27] during exponential PCR as follows:

(1) delta Ct (dCt) = test mRNA Ct value – Ct values for housekeeping mRNA

(2) delta–delta Ct (ddCt) = siRNA-treated dCt – mock or control oligo dCt (this number should be positive for mRNA knock-down)

(3) Fold change =  $2^{-ddCt}$

(4) Per-cent control = fold change \* 100%

Samples were run in duplicate, and per-cent control  $\pm$  standard deviation were reported. Significance was determined using the Mann–Whitney test.

**siRNA KD/degranulation assays.** RBL-2H3 cells were transfected with siRNA ( $1 \times 10^6$  cells/transfection) as described above and split into six wells of a 24-well plate in 500 µl/well of complete media with 0.3 µg/ml anti-DNP IgE. After 24 h, degranulation was initiated with 100 ng/ml BSA-DNP in 300 µl of HEPES-buffered saline. The assay was continued as described above.

**Histamine. ELISA** Histamine in cell supernatants was measured via ELISA (IBL cat# IB89143; Minneapolis,

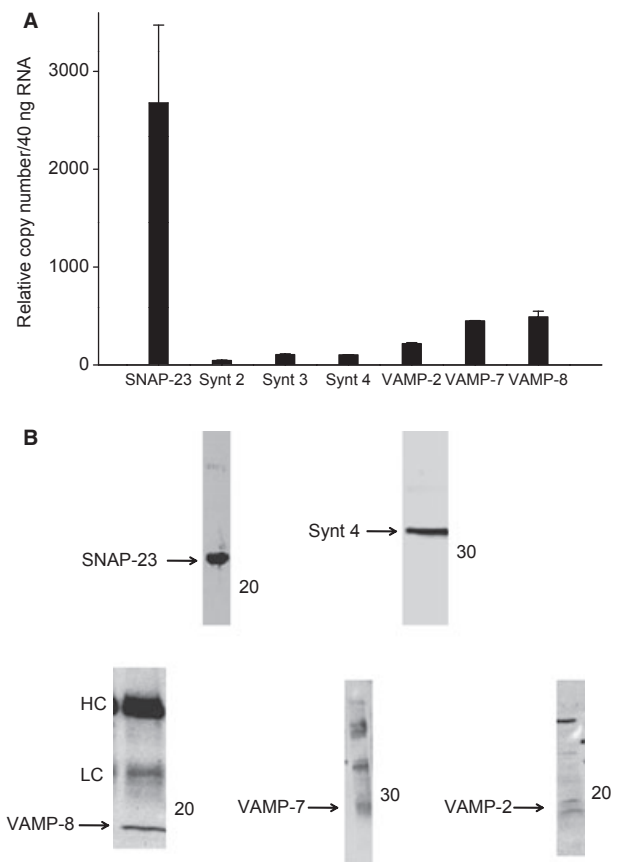


**Figure 1** Relative mRNA expression of SNARE proteins in RBL-2H3 cells. (A) Relative copy number of various SNARE mRNA was determined for 2 µl (40 ng) of cDNA generated from 1 µg of RBL-2H3 total RNA. Copy number was calculated using a rat  $\beta$ -actin standard curve. Samples are the mean of two separate Taqman samples  $\pm$  standard deviation. (B) Protein expression (SNAP-23, VAMP-7, VAMP-2) was determined via Western blot in triton X-100 lysates of RBL-2H3 cells. Twenty-five microlitres of cleared lysate was run per lane from approximately  $2.5 \times 10^7$  cells/ml. Alternatively (Syntaxin 4, VAMP-8), lysates were immunoprecipitated with polyclonal antibodies and protein G agarose, and then eluates were blotted as described. HC, immunoglobulin heavy chain; LC, immunoglobulin light chain.

MN, USA) according to the manufacturer's instructions. Supernatants and lysates from degranulation experiments described above were diluted 1:2 into the ELISA, and histamine levels were extrapolated off a standard curve.

## Results

### SNARE target identification and protein level determination

In order to identify the appropriate mast cell molecular targets for siRNA inhibition, we assessed the RNA and protein levels of particular SNARE family members utilizing quantitative real-time PCR on cDNA reverse-transcribed from mRNA isolated from the rat mast cell line, RBL-2H3. In Fig. 1A, we show the relative

expression of SNAP-23 (the major non-neuronal SNAP family member) and various members of the VAMP and Syntaxin families. SNAP-25, the predominant neuronal SNARE, was not detected by RT-PCR in RBL-2H3 cells (data not shown). The VAMP family mRNAs expressed in RBL-2H3 cells are VAMP-7 and VAMP-8, with slightly lower expression of VAMP-2. The Syntaxin family members are expressed at relatively low levels with approximately equal relative expression of Syntaxin 3 and Syntaxin 4.

SNARE protein levels were determined by Western blot analysis of triton X-100 lysates or immunoprecipitates. Data shown in Fig. 1B demonstrate that SNAP-23 is the SNAP family SNARE representative expressed in RBL-2H3 cells. SNAP-25 was not detected in RBL-2H3 lysates. The only detectable Syntaxin SNARE protein expressed in RBL-2H3 cells is Syntaxin 4, as an antibody to Syntaxin 1 did not detect a band in RBL-2H3 lysates but did work on a rat brain extract (data not shown). Antibodies to Syntaxin 3 and Syntaxin 2 did not detect any protein in RBL-2H3 lysates, but were not validated with a positive control lysate (data not shown). VAMP-2, VAMP-7 and VAMP-8 are the VAMP family SNARE members expressed in RBL-2H3 cells.

Functional SNARE complexes formed after RBL-2H3 activation were immunoprecipitated using anti-SNAP-23-conjugated protein G agarose. Cells were pretreated with *N*-ethylmaleimide (NEM), an NSF inhibitor, to preserve SNARE complex formation. Cells were then activated for 5 min in the presence of 1  $\mu$ g/ml ionomycin and subsequently lysed, and immunoprecipitations were carried out as described. Syntaxin 4 and VAMP-8 co-precipitate with SNAP-23 in activated RBL-2H3 lysates

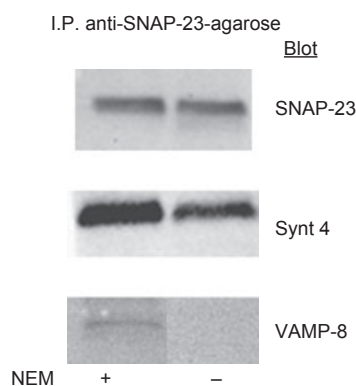


**Figure 2** Co-precipitation of VAMP-8 and Syntaxin 4 with synaptosomal-associated protein (SNAP)-23. Immunoprecipitation of RBL-2H3 lysates with anti-SNAP-23-conjugated protein G agarose. Pulldown of SNAP-23, Syntaxin 4 and VAMP-8 protein in the absence/presence of 1 mM *N*-ethylmaleimide pretreatment. Membranes were blotted with anti-SNAP-23, anti-Syntaxin 4 and anti-VAMP-8 polyclonal serum and a goat-anti-rabbit HRP conjugate as described in Materials and methods.

**Table 1** List of siRNAs employed in SNARE knock-down experiments.

| siRNA          | Supplier   | Catalogue number     |
|----------------|------------|----------------------|
| Rat VAMP-8     | Invitrogen | 127849A04-05, 07-010 |
| Rat VAMP-2     | Ambion     | S128460              |
| Rat SNAP-23    | Dharmacon  | LU-093035-01         |
| Rat Syntaxin 4 | Invitrogen | 121307A01-A06        |
| Rat Syk        | Ambion     | AM16708              |
| Rat VAMP-7     | Ambion     | S137023              |
| Rat GAPDH      | Dharmacon  | GAPDH control        |

SNAP, synaptosomal-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

(Fig. 2). NEM treatment is required to detect VAMP-8, while NEM treatment increases overall Syntaxin 4 detection. VAMP-2 and VAMP-7 did not co-precipitate with SNAP-23 with or without NEM treatment (data not shown). These data suggest that SNAP-23, VAMP-8 and Syntaxin 4 form the major SNARE complex in activated RBL-2H3 mast cells.

#### siRNA knock-down trials

Utilizing the SNARE complex information identified above, we began the process of quantifying target efficacy for siRNA interference-mediated inhibition of function utilizing synthetic RNAi oligos (siRNA) to knockdown SNARE mRNA and subsequent protein expression (see Table 1 for siRNA list). We determined the effect on degranulation resulting from this knock-down in an *in vitro* degranulation assay that detects the release of  $\beta$ -hexosaminidase, an enzyme marker of secretory granules and lysosomes that parallels the release of mast cell inflammatory mediators.

Several methods were tested to assess overall RBL-2H3 transfection efficiency. We used liposome-based transfection methods (RNAiMax and Dharmafect buffers) with different serum conditions/time courses and evaluated various electroporation methods (AMAXA Nucleofection). Our trials identified a Nucleofection protocol [26] that resulted in high transfection efficiencies (>85%) and allowed the use of serum during transfections providing low cell death, post-transfection. This protocol was utilized throughout our studies. We then used a siRNA to rat GAPDH to validate our transfection protocols. Quantitative real-time PCR allowed us to determine mRNA levels in RBL-2H3 cells treated with control siRNA or a rat GAPDH-specific siRNA. siRNA specific for rat GAPDH was able to knock down GAPDH mRNA, while a control oligo did not have an effect on GAPDH mRNA (Fig. 3).

We then went ahead and treated RBL-2H3 cells with a siRNA specific for rat SNAP-23. siRNA to rat SNAP-23 specifically knocks down SNAP-23 mRNA with a

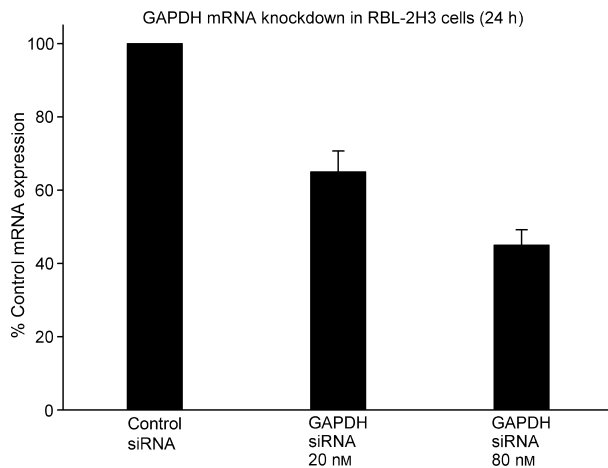


**Figure 3** Knock-down of GAPDH mRNA in siRNA-treated RBL-2H3 cells. Knock-down of GAPDH mRNA after 24 h treatment with 20 and 80 nM siRNA specific for GAPDH. Percent control mRNA expression was calculated as described in Materials and methods, and control siRNA was set as control. Values are the mean of two replicates  $\pm$  the standard deviation. Shown is a representative experiment of 2.

maximal inhibition at 40 nM (Fig. 4A). To analyse if SNAP-23 protein was also decreased in our siRNA-treated cells, we performed transfections with 40 nM SNAP-23 or control siRNA at various timepoints; cells were lysed and Western blots were performed to identify both actin and SNAP-23 expression. In Fig. 4B, we demonstrate that SNAP-23 siRNA, but not control siRNA, reduced SNAP-23 protein expression and that protein reduction appears optimal at 24 h as SNAP-23 protein levels start to return to baseline in a time-dependent fashion. Using spot densitometry (as described in Materials and methods), we estimate that relative to actin, SNAP-23 protein levels decrease by approximately 76% at 24 h post-transfection. We then chose 24 h for our subsequent SNARE knock-down and degranulation experiments in RBL-2H3 cells. Next, we treated RBL-2H3 cells with a VAMP-8 siRNA and again checked for both mRNA knock-down and protein reduction at 24 h with 40 nM control or VAMP-8-specific siRNA. As shown in Fig. 5A, B, both VAMP-8 mRNA and protein were knocked down after siRNA treatment at 24 h. Using spot densitometry, VAMP-8 protein knock-down at 24 h was close to approximately 90%.

Before determining the effect of SNARE siRNA-mediated knock-down in antigen-induced degranulation assays, we validated the use of siRNA in RBL-2H3 cells with a positive control. We utilized a Syk kinase siRNA, a specific, well-validated proximal signalling molecule pivotal in IgE-induced mast cell degranulation. Figure 6 shows that 40 nM of a siRNA designed to knock down Syk inhibited antigen-induced degranulation by approximately 60%. Individual experiments resulted in inhibitions ranging from approximately 50% to more than

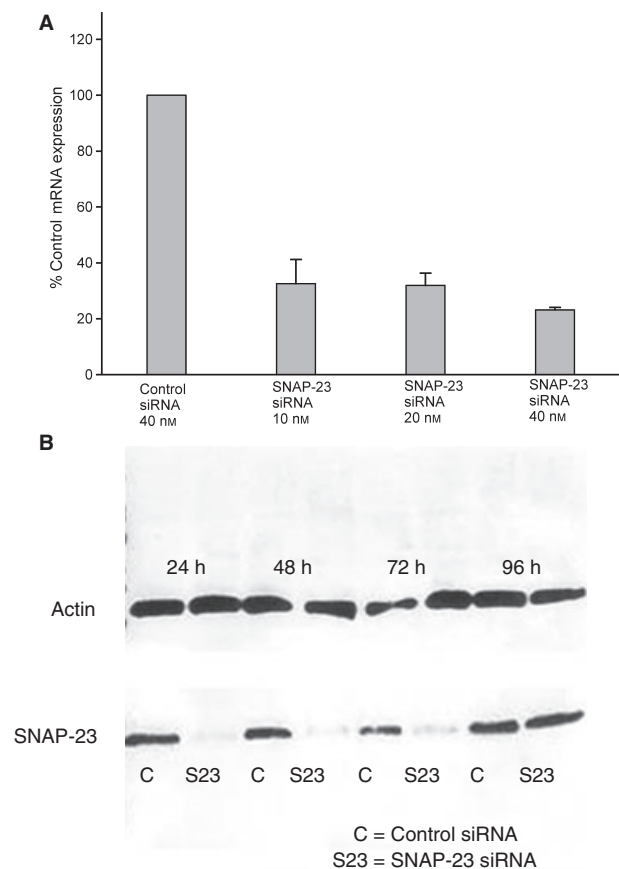


**Figure 4** Knock-down of synaptosomal-associated protein (SNAP)-23 mRNA and protein levels in RBL-2H3 cells after treatment with SNAP-23 siRNA. (A) Knock-down of SNAP-23 mRNA after 24 h treatment with 10, 20 and 40 nM siRNA specific for SNAP-23. Percent control mRNA expression was calculated as described in Materials and methods, and control siRNA treatment was set as 100%. Values are the mean of two replicates  $\pm$  standard deviation. Shown is a representative experiment of 3. (B) Knock-down of SNAP-23 protein levels in RBL-2H3 cell lysates after 24, 48, 72 and 96 h treatment with 40 nM SNAP-23 siRNA. Lysates were blotted with anti-SNAP-23 and anti-actin, and relative SNAP-23 protein levels were determined by normalization to actin bands and comparison to control siRNA-treated samples as described in Materials and methods. Shown is a representative experiment of 3.

90% ( $n = 4$  transfections; 13 total replicates). Syk protein knock-down approached approximately 80% [26]. After validation with Syk, we tested both SNAP-23 and VAMP-8 siRNA in our degranulation assays using 40 nM siRNA (no increased effect was noticed at  $>40$  nM) and at 24 h post-transfection. In Fig. 6, we show that SNAP-23 and VAMP-8 siRNA treatment resulted in a significant decrease in antigen-induced degranulation with inhibitions approaching approximately 30% and 50%, respectively. Replicate experiments showed similar results (SNAP-23;  $n = 5$  transfections; 22 total replicates/VAMP-8;  $n = 4$  transfections; 20 total replicates). siRNA to Syntaxin 4 was then utilized to evaluate its role

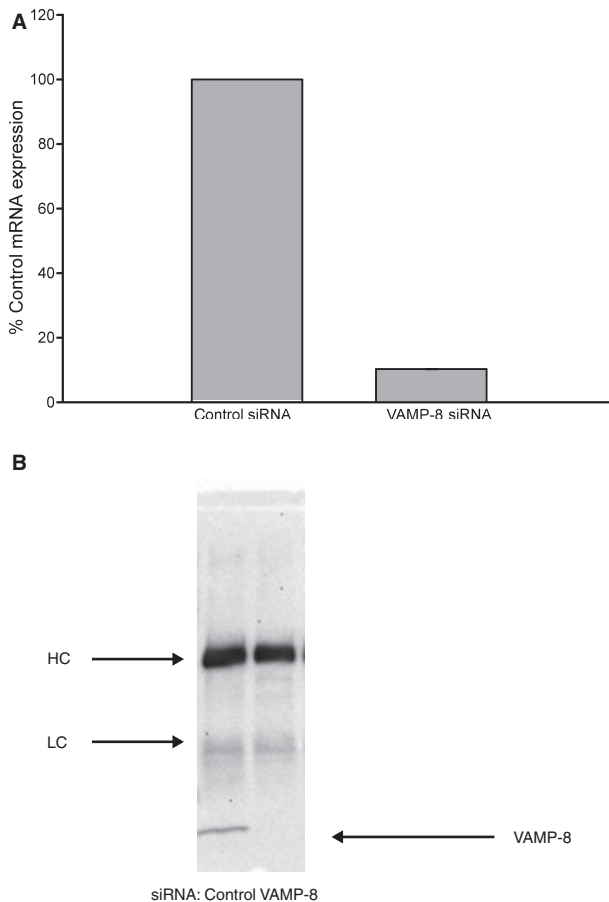


**Figure 5** Knock-down of VAMP-8 mRNA and protein levels in RBL-2H3 cells after treatment with VAMP-8 siRNA. (A) Knock-down of VAMP-8 mRNA after 24 h treatment with 40 nM siRNA specific for VAMP-8. Percent control mRNA expression was calculated as described in Materials and methods, and control siRNA treatment was set at 100%. Values are the mean of two replicates  $\pm$  standard deviation. Shown is a representative experiment of 2. (B) Knock-down of VAMP-8 protein levels in RBL-2H3 cell lysates after 24 h treatment with 40 nM VAMP-8 siRNA. Lysates were immunoprecipitated with anti-VAMP-8 polyclonal serum/protein G agarose, and eluates were then blotted with anti-VAMP-8. Lysates were also blotted with anti-actin antibody, and relative VAMP-8 protein levels were determined by normalization of VAMP-8 bands to actin bands and then comparison to control siRNA-treated samples as described in Materials and methods.

in degranulation. Syntaxin 4 siRNA treatment (40 nM, 24 h) resulted in a significant decrease in antigen-induced degranulation (approximately 30%) (Fig. 6). Replicate experiments confirmed initial results ( $n = 3$  transfections; 15 total replicates). Syntaxin 4 protein could only be knocked down approximately 35% at 24 h as visualized by Western blot and spot densitometry (data not shown). Since VAMP-7 has been recently shown to be present in complexes with SNAP-23 and Syntaxin 4 and implicated in mast cell degranulation [20, 28], we examined whether VAMP-7 targeting would affect degranulation (Fig. 6). Inhibition of VAMP-7 expression results in a decrease in

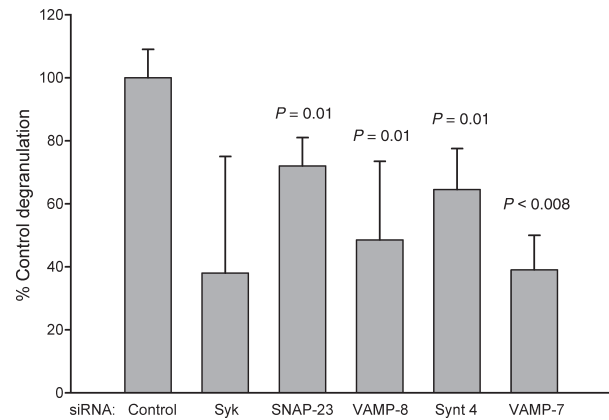


**Figure 6** Inhibition of antigen-induced degranulation in RBL-2H3 cells by siRNA specific for Syk, synaptosomal-associated protein (SNAP)-23, VAMP-8 and Syntaxin 4. Inhibition of antigen-induced degranulation in RBL-2H3 cells treated for 24 h with 40 nM of Syk siRNA. Representative of four individual transfections; 13 total replicates. SNAP-23: Inhibition of antigen-induced degranulation in RBL-2H3 cells treated for 24 h with 40 nM of SNAP-23 siRNA. Representative of five individual transfections; 22 total replicates. VAMP-8: Inhibition of antigen-induced degranulation in RBL-2H3 cells treated for 24 h with 40 nM of VAMP-8 siRNA. Representative of four individual transfections; 20 total replicates. Syntaxin 4: Inhibition of antigen-induced degranulation in RBL-2H3 cells treated for 24 h with 40 nM of Syntaxin 4 siRNA. Representative of three individual transfections; 15 total replicates. VAMP-7: Inhibition of antigen-induced degranulation in RBL-2H3 cells treated for 24 h with 40 nM of VAMP-7 siRNA. Representative of two individual transfections; 10 total replicates. *P*-values were determined using the Mann-Whitney Test and calculated comparing control siRNA-treated cells to target siRNA-treated cells.

antigen-induced degranulation that routinely approached approximately 40% ( $n = 2$ ; 10 total replicates), thus implicating VAMP-7 and VAMP-8 as important players in antigen-mediated degranulation. Knock-down of VAMP-2 mRNA with a VAMP-2-specific siRNA did not modulate RBL-2H3 degranulation (data not shown). These results suggest that SNAP-23 and Syntaxin 4, complexed with VAMP-7 or VAMP-8, form ternary complexes that mediate RBL-2H3 degranulation.

In addition to  $\beta$ -hexosaminidase, we also characterized the release of the more physiologically relevant mediator histamine from activated RBL-2H3 cells treated with control or SNARE-targeted siRNA. Inhibition profiles for histamine release for the target siRNAs (Fig. 7A) correlated well with the  $\beta$ -hexosaminidase further supporting the role for these SNAREs in RBL-2H3 degranulation.

## Discussion

Data from the results are summarized in a model of the relevant SNARE targets responsible for activation-induced degranulation in RBL-2H3 mast cells (Fig. 7B). This model is based on expression levels, co-immunoprecipitation results and functional degranulation studies

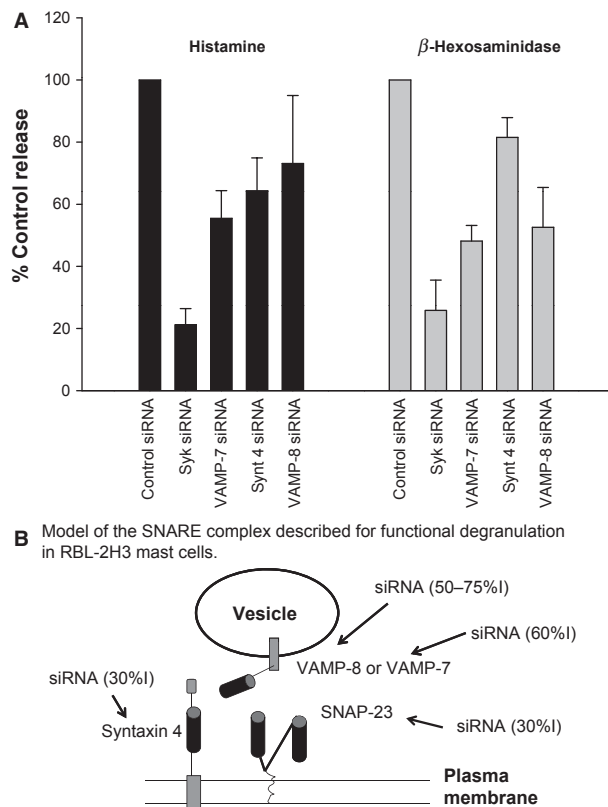


**Figure 7** Histamine/ $\beta$ -hexosaminidase levels correlate after siRNA knock-down. Proposed model of the functional soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex mediating mast cell degranulation. (A) Inhibition of antigen-induced degranulation (as measured by histamine release and  $\beta$ -hexosaminidase activity) in RBL-2H3 cells treated for 24 h with 40 nM of Syk, Syntaxin 4, VAMP-8 or VAMP-7 siRNA. Data represent the mean of 10 replicates from two individual transfections. (B) We propose that synaptosomal-associated protein (SNAP)-23 and Syntaxin 4 present on the plasma membrane interact to form a binary complex contributing SNAP and Syntaxin family SNARE motifs to the functional core complex. Upon stimulation via antigen/IgE binding and subsequent calcium influx, this binary complex interacts with the vesicle SNAREs, VAMP-8 or VAMP-7 to form a ternary complex that mediates fusion and degranulation.

and suggests that SNAP-23, Syntaxin 4 and both VAMP-7 and VAMP-8 form a set of ternary complexes that mediate activation-induced degranulation in RBL-2H3 mast cells.

The 'proof of principle' that SNARE proteins can be targets for therapeutic intervention comes from the success of *Botulinum* toxin type A (Botox<sup>TM</sup>), approved for cosmetic applications and for the treatment of spasms and dystonias. *Botulinum* toxins have a transient inhibitory effect on muscles, decreasing their ability to contract. The canonical neuronal SNARE complex (SNAP-25, Syntaxin 1 and VAMP-2) are targets of the *Botulinum* proteases and mediate neurotransmitter-laden vesicle docking/fusion and subsequent neurotransmitter release in the central nervous system [29, 30]. Bioinformatics data suggesting homologous SNARE protein

expression in secretory cells outside the CNS (such as mast cells) have led us to investigating the possibility of targeting SNAREs specifically involved in mast cell degranulation for the treatment of allergic and autoimmune disease.

The observed expression of SNAP-23, Syntaxin 4, VAMP-7, VAMP-2 and VAMP-8 mRNA and protein in RBL-2H3 cells presented in this article is in agreement with previous work [11, 12]. Using immunoprecipitation (IP) with an anti-SNAP-23 antibody, we were able to pull down Syntaxin 4 (Fig. 2) and VAMP-8 (only after NEM treatment), but not VAMP-2 or VAMP-7. In human mast cells, Sander *et al.* [28] showed that SNAP-23 co-precipitated with Syntaxin 4, VAMP-7 and VAMP-8, but not VAMP-2 and VAMP-3. While we did not observe any association of VAMP-7 in our co-precipitates, it may still be possible that VAMP-7 is present in a complex with SNAP-23 and Syntaxin 4. As we did not detect a robust VAMP-8 signal in co-precipitates even after NEM treatment, it is possible that given our current sensitivity, we are missing VAMP-7. Several reports have demonstrated that activation-induced ternary SNARE complexes are notoriously transient and may be very difficult to 'capture' during immunoprecipitation studies [31, 32].

No small-molecule inhibitors of mast cell SNAREs have been identified to date. Additionally, as SNAREs are intracellular targets, the use of biological entities such as recombinant proteins or neutralizing antibodies is not possible. Therefore, interfering RNA offers the best approach to develop a SNARE-directed therapy. To date, several siRNA-based therapies have initiated clinical trials for the treatment of viral diseases, cancer and macular degeneration [23]. Using siRNA modulation, we achieved a 50% knock-down in RBL-2H3 degranulation by targeting VAMP-8. Interestingly, Tiwari *et al.* [21] demonstrated an *in vitro* degranulation defect in mast cells in VAMP-8 knockout mice that approached 50%. This decrease translated *in vivo* to a significant effect in plasma histamine upon antigen challenge in a passive cutaneous anaphylaxis model. These studies help validate the efficacy and predictability of the *in vitro* degranulation assay. Additionally, our siRNA data identify SNAP-23 and Syntaxin 4 as important for RBL-2H3 degranulation, and knock-down of these SNAREs resulted in 30% inhibition of degranulation when compared to control siRNA treatment. In our best-case scenarios, we observed a maximum of 80–90% decrease in mRNA and protein levels. It is possible that the remaining 10% of the mRNA and protein can still contribute to a large percentage of the degranulation activity, although it may be possible to re-treat cells with siRNA in order to decrease the mRNA/protein levels. Our data suggest that the effect of siRNA in RBL-2H3 cells is transient as protein levels start to return after 48 h post-

transfection and are back to normal by 96 h. Our data accurately reflect the effect of SNARE knock-down, as we utilized whole cells stimulated with physiological stimuli. Interestingly, a recent report [33] suggests that due to SNARE redundancy and the apparent reserve of SNARE expression in cells, it may be difficult to detect an overt phenotype in siRNA-treated cells. We have utilized robust transfection/assay methodologies to detect a consistent degranulation phenotype in RBL-2H3 cells.

*In vitro* degranulation assays represent a model system for the validation of SNARE siRNAs that modulate mast cell activation. As we progress to *in vivo* models of allergy and autoimmune disease, a variety of delivery methods must be validated to ensure proper coverage to optimize efficacy of drug. This remains a major challenge in the field of siRNA-based therapeutics. While nucleofection and liposome technologies are effective in our *in vitro* siRNA studies in RBL-2H3 cells, *in vivo* delivery of siRNAs to target tissues is more challenging. Before choosing a siRNA for *in vivo* studies, a panel of oligos should be screened for potency and for off-target effects in *in vitro* assays and gene-chip assays. These 'qualifying' measures are necessary to increase the chance of reproducing efficacy *in vivo* while reducing off-target phenotypes (e.g. toxicity).

Our studies indicate that siRNA is the most practical method to regulate the SNARE complex's contribution to degranulation in anaphylaxis and autoimmune disease. This study demonstrates the efficacy siRNA has in modulating the functional SNARE degranulation complex. Additional studies focusing on *in vivo* delivery of the siRNAs identified above will further validate the use of siRNA for the treatment of allergic and autoimmune disease.

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