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C. elegans v-SNARE master protein 1 (VSM-1) fails to co-precipitate with neuronal SNARE proteins SNB-1, UNC-64, and RIC-4A/B during *in vitro* pull downs

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Abstract

In the cell body neurotransmitters are released extracellularly by vesicle exocytosis, which are mediated via proteins called SNAREs. Moreover, work from the Jeffrey Gerst laboratory demonstrated that Vsm1 is a v-SNARE binding protein that inhibits exocytosis in yeast. In particular, they showed that in *Saccharomyces cerevisae* Vsm1 mutants have increased protein secretion, while the overexpressors showed inhibition of secretion and cell growth. Using structural protein analysis, they also established that an intact retroviral protease (RVP) domain found in Vsm1 is essential for the regulation of constitutive secretion in yeast. To further investigate the modulatory role of Vsm1 and test whether it acts in calcium-mediated exocytosis, our laboratory began characterizing the *C. elegans* homolog named VSM-1. Examination of the amino acid sequences shows that the RVP domain found in yeast Vsm1 is conserved from nematode to humans. Based on this initial finding, we hypothesize that in multicellular organisms such as *C. elegans,* VSM-1 may bind and cleave neuronal v-SNARE synaptobrevin (SNB-1) as well as t-SNAREs syntaxin (UNC-64) and SNAP25 (RIC-4), thereby inhibiting calcium mediated, SNARE dependent synaptic vesicle exocytosis. To test this hypothesis, our group began investigating if these SNARE proteins are VSM-1 binding partners and what role the VSM-1 RVP domain plays in this interaction. To this effect, a battery of recombinant DNA plasmids coding for His-tagged SNB-1, UNC-64, RIC-4A and RIC-4B as well as a variety of GST fused VSM-1 were generated using conventional molecular biology techniques and Site-Directed-Mutagenesis approaches. Next, His-tagged SNARE proteins, GST fused full length wild type VSM-1, GST fused full length VSM-1 containing a point mutation in the RVP active site, GST fused VSM-1 lacking the N terminus and GST alone were affinity purified. Once the proteins were obtained, *in vitro* binding and cleavage assays were performed and analyzed by SDS-PAGE and gel densitometry. *In vitro* binding assays reveal that none of the SNARE proteins co-precipitated with VSM-1. Analyses of cleavage studies via SDS PAGE and HPLC, show no apparent reduction of the size of SNB-1, deducing that SNB-1 is not proteolyzed by the RVP domain of VSM-1. To conclude, analysis of *C. elegans* VSM-1 shows that neuronal SNARE proteins UNC-64, RIC-4A, RIC-4B, and SNB-1 do co-precipitates with VSM-1. Furthermore, it was determined that SNB-1 does not appear to be the substrate for the RVP catalytic activity of VSM-1.

Keywords: Exocytosis, Proteolysis, Vsm1/VSM-1, SNAREs

1. Introduction

Neuronal exocytosis is a highly regulated process mediated by the formation of trimeric SNARE complexes. These SNARE complexes operate in a zipper like fashion [1] where, a vesicle SNARE (v-SNARE) synaptobrevin (called SNB-1 in *C. elegans*) binds to target SNAREs (t-SNARE) syntaxin (UNC-64), and SNAP25 (RIC-4A and RIC-4B) [2-4]. The resulting SNARE complex runs laterally to the plasma membrane and its formation pulls the opposing membranes into close proximity where a fusion pore develops between the two membranes [4-6]. A putative

Ca^{2+}sensing protein termed synaptotagmin is believed to interact with vesicles anchored via SNARE complexes, regulating the transition of vesicle priming to vesicle fusion in a calcium dependent manner [7]. This Ca^{2+} sensitive protein mediates the vesicle fusion event upon a rise in intracellular Ca^{2+} concentration through a mechanism that is not fully understood [7,8]. The formation of SNARE complexes is a multifaceted and tightly regulated process involving many key regulators [9]. Thus, the focus of this research report is to expand our knowledge on one candidate for this task, the protein VSM-1.

Past research using yeast secretion mutants led to the identification of Vsm1 (VSM-1 in *C. elegans*), a soluble protein that was shown to bind to t- and v-SNARE proteins [10]. Analysis of Vsm1 amino acid sequence uncovered 4 protein domains, RVP (retroviral protease domain), UBL (ubiquitin-like domain), UBA (ubiquitin associated domain), and PEST (a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T) amino acids) [11]. Moreover, studies of protein structure-function show that these domains have an important role in the regulation of the cell cycle, membrane trafficking and SNARE-mediated secretion [11]. Cells overexpressing yeast Vsm1 are characterized by diminished cell growth, an accumulation of secretory vesicles and a reduction of protein secretion [10]. Investigations on the molecular mechanisms underlying the Vsm1 overexpressor phenotype also show that Snc1 and Snc2 (the yeast homologs for synaptobrevin) immunoprecipitate with Vsm1 [11]. Yet in another *in vitro* immunoprecipitation binding assay, Gerst and Marash demonstrated that Vsm1 binds to Sso1 (the yeast syntaxin homolog) and displaces Sec9 (the yeast SNAP25 homolog) in a competitive fashion [12]. Further reports show that phosphorylation of a Serine residue located at position 79 of Sso1 by PKA (Protein Kinase A) increases Sso1's affinity for Vsm1 by a factor of five [12]. Together these findings suggest that Vsm1 has the potential to inhibit assembly of SNARE complexes by blocking binding of Sec9 with Sso1 and thus membrane fusion.

Recent work from our laboratory focusing on the study of *C. elegans* VSM-1 suggests that it is likely to play a similar role to the yeast counterpart. More importantly, studies performed by our group demonstrated that in worms, multicellular organisms possessing a nervous system, VSM-1 expresses in multiple tissues including muscles and nerve cells (unpublished work). Phenotypic examinations of null VSM-1 nematode mutants identify muscle hyperactivity, increased synaptic release of acetylcholine (ACh) and enhanced synaptic density, all indicative of VSM-1's negative role in neuronal function.

To further investigate *C. elegans* VSM-1 mode of action and gain insights into its regulatory role in neurons, we began a detail analysis of protein binding assays and proteolysis. To this end, we designed recombinant protein and *in vitro* binding assays involving neuronal-specific SNARE proteins and various VSM-1 full length and truncated versions. Results shown in this report suggest that the nematode VSM-1 protein does not co-precipitates with the neuronal SNARE proteins UNC-64, SNB-1 and RIC-4 isoforms A and B. Incubation of VSM-1 featuring a retroviral aspartic proteinase domain rules out a potential role cleaving the v-SNARE protein SNB-1.

2. Materials and Methods

2.1 His-tagged recombinant DNA plasmids:

SNB-1 (pH04sb61-62) and UNC-64 (pRSETA) constructs were kindly provided by Dr. Mike Nonet, Washington University, St. Louis, MO. SNAP25/RIC-4A and RIC-4B constructs were engineered using conventional molecular biology tools and techniques. In brief, a Qiagen plasmid DNA miniprep was performed to isolate and purify plasmids PQE-32, pRSETC (RIC-4B) and QSNP/pRSETC (RIC-4A) (the last two plasmids were kindly provided by Dr. Mike Nonet). Restriction enzymes *Hind*III and *BamH*I (New England BioLabs) double digest was utilized to linearize the PQE-32 vector and produce the RIC-4A and RIC-4B inserts. Digested DNA fragments were separated using gel electrophoresis and gel extracted using QIAquick Gel Extraction Kit (Qiagen). Excised DNA fragments were then ligated via a T4 Ligase (New England Biolabs) following the manufacturer recommendations and producing ah126 (RIC-4A) and ah127 (RIC-4B). Ligation reactions were used to transform TOP10 Chemically Competent *E. coli* cells (Invitrogen Life Technologies), which were plated onto luria broth (LB) agar plates supplemented with ampicillin (100 µg/mL) for overnight growth at 37°C. Before sequencing plasmids, an *Eco*RI (New England BioLabs) analytical digest was performed for preliminary verification.

2.2 GST-tagged recombinant DNA plasmids:

Before producing GST plasmids containing VSM-1 cDNA sequences, reverse transcription polymerase chain reactions (RT-PCR) were performed. First, RNA templates isolated from wild-type *C. elegans* nematodes were

used to generate single stranded cDNA using the Protoscript First Strand cDNA synthesis kit (New England BioLabs). 2µg of total RNA was converted to cDNA utilizing the manufacturer recommended protocol and primer ahluc18 5'-CTCGAGGGCGGAACTATGTGGA-3' (Sigma-Aldrich). Next, a PCR reaction was performed using primer ahluc18 and ahluc17 5'-GTCGACGTGAGCGTAGCCGTTA-3' (Sigma-Aldrich). GoTaq Green Master Mix (Promega) was added in a final concentration of 1X containing 25 units/mL of Taq DNA Polymerase in a buffer of pH 8.5, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, and 1.5 mM $MgCl_2$. A standard PCR program was used to amplify our templates. PCR amplicons were then cleaned using the PCR Purification Kit (QIAquick) and manufacturer recommendations. Purified PCR fragment containing the coding sequence for VSM-1 was cloned into TOPO TA vectors (Invitrogen) producing plasmid ah102. TOPO cloning reactions were transformed into chemically competent TOP10 *E. coli* cells and plated on LB agar, 160 µg of *X-gal*, 50 µg/mL of Kanamycin, and 0.16 mM IPTG to perform blue/white selection. Last, ah102 and pGEX-4T3 (Invitrogen) plasmids were double digested with *Sal*I and *Xho*I (New England BioLabs) and fragments ligated to generate ah103 (full length VSM-1 fussed to GST). Plasmid ah103 and pGEX-4T1 (Invitrogen) were double digested with *Xho*I, *EcoRI* and fragments ligated to generate ah115 (delta N terminus VSM-1 fused to GST). Site directed mutagenesis (Stratagene) using primers 5'-CCAGTCAAAGCTTTCATTGCTTCTGGA GCTCAAAAATCG-3' and 5'-CGATTTTTGAGCTCCAGAAGCAATGAAAGCTTTGACTGG-3' (Sigma-Aldrich) were used together with plasmid ah103 to produce full length mutant VSM-1 (D261A) fused to GST.

2.3 Batch affinity purification of recombinant proteins:

Recombinant DNA plasmids containing sequences for SNARE proteins and VSM-1 were transformed into BL21 *E. coli* strains and grown overnight at 37˚C on LB agar plates supplemented with 100 µg/mL ampicillin. Isolated bacterial colonies grown on selective media were used to inoculate 5 mL 2X YT liquid cultures supplemented with 100 µg/mL ampicillin. One liter of 2X YT was prepared mixing 16 g Tryptone (IBI Scientific), 10 g yeast extract (IBI Scientific), and 5 g NaCl. Liquid cultures were then incubated overnight at 37˚C shaking at 250 rpm. The next day each culture was diluted 1/50 in 2X YT with 100 µg/mL ampicillin, and incubated at 37°C, 250 rpm shaking until a 0.6-0.8 optical density at 600 nm was reached. After the necessary optical density had been attained, IPTG was added to the final concentration of 0.5 mM, and cultures were grown for 2- 4 hours. Last, cultured cells were harvested in bottles using a centrifugation of 5,000 x g for 10 min. at 4°C. Pelleted cells were stored at -80°C until use.

For protein affinity purifications, pelleted cells were resuspended in 5 mL of 1X PBS (Sigma) containing 1X Halt tm Protease Inhibitor Single-use Cocktail (Thermo Scientific). Bottles were vortexed to loosen the pellets and periodically cooled on ice to prevent protein degradation. The resuspended cells were transferred into separate 15 mL tubes and lysed via sonicator with a series of three 30 sec bursts at 60 amps followed by 30 sec incubation periods on ice between each burst. A solution of 1% Triton X-100 in PBS was added to resuspended cells and incubated shaking for 20 min at 4°C. After the incubation period, soluble cell lysate was obtained via centrifugation at 3,700 x g for 20 min at 4°C. In the meantime, a tube containing 2 mL of 50% Ni-NTA slurry (Qiagen) per 250 mL of initial bacterial culture was centrifuged for 3 min at 3,700 x g, 4°C. The supernatant was decanted and clean beads were kept on ice. Once the cell lysate was cleared by centrifugation, the supernatant was transferred into the tubes containing beads and bound overnight at 4°C on a rotary shaker. The next day, the bead-batch affinity purification mixture was centrifuged at 4°C at 3,700 x g for 3 min. and pellets were washed 5 times for 5 min rocking at 4°C with 10 mL of Ni-NTA wash buffer (pH 8.0, 50 mM NaH_2PO_4 (Sigma-Aldrich), 300 mM NaCl (Fisher), and 20 mM Imidazole (Sigma-Aldrich)). Each wash was followed with a 2 min centrifugation at 3,700 x g, 4°C. Affinity purified tagged proteins were then eluted once with 0.5 mL elution buffer for 2 hours at 4°C.

GST fusion proteins were affinity purified using a similar technique, except that cells were resuspended in 5 mL 1X PBS, 1 X Halt tm Protease Inhibitor Single-use Cocktail (Thermo Scientific), and 1X EDTA prior to lysing. Lastly, crude extract was transferred into tubes containing Glutathione Sepharose beads (General Electric Healthcare) and affinity purified GST fusion proteins were not eluted from beads.

2.4 SDS-PAGE:

Electrophoresis samples were prepared by adding 4X XT/ 20X Reducing agent solution (Bio-Rad) to make a final concentration of 1X each. The samples were heated to 70°C for 5 minutes and stored at -20°C until ready for use. Aliquot samples of 20 µL were loaded into Criterion XT Precast Gels (Bio-Rad) and ran for 1 hour at 125 volts using an XTMes buffer system (Bio-Rad). Once the gels had run for 1 hour, they were stained for 1 hour using

Coomassie Brilliant Blue R250 staining solution (Bio-Rad). Last, Coomassie Brilliant Blue R250 Destaining Solution was used for overnight destaining and gels were imaged-analyzed using the ChemiDoc XRS+ system and image lab software (Bio-Rad). Protein concentration was determined by quantifying the density of Coomassie Brilliant Blue stain in affinity purified protein samples and BSA (Bovine Serum Albumin) standards ranging from 80-1000 ng. Standards and other protein samples run on the same gel were imaged using image lab software (Bio-Rad).

2.5 *In Vitro* Binding Assays:

Varying amounts of eluted His-tagged SNARE proteins (see figures 1 and 2) were combined with sepharose beads containing 26-28 μg of GST, ΔNT VSM-1 (delta amino terminus VSM-1), or FL VSM-1 (full length VSM-1) and up to 500 μL of 0.5% NP40 in 1X PBS buffer. Binding reactions were incubated overnight at 4°C. The following day, beads were centrifuged at 3,700 x g, 4°C for 5 min. Beads were then washed 5 times with 10 mL of 0.5% beta-mercaptoethanol/NP40 in 1X PBS buffer. Finally, a mixture of 4X XT/ 20X Reducing Agent buffer (Bio-Rad) was added to each sample to reach a final concentration of 2X. Samples were heated at 70°C for 5 min and 20 μL samples were loaded into 4-12% Criterion XT Precast Gels (Bio-Rad). PAGE was ran using 1X XTMes buffer solution for 1 hour at 125 V. Gels were then stained for 1 hour and then destained overnight using the Coomassie Brilliant Blue R250 staining and destaining solutions (Bio-Rad). Last, gels were imaged using a ChemiDoc XRS+ system and image lab software (Bio-Rad). Quantification was performed using the manufacturer recommendations.

2.6 Cleavage Assays:

Proteinase cleavage assays were prepared in either acidic (Buffer A: pH 3.5, 50 mM NaCl, 20 mM NaAcetate) or neutral (PBS pH 7.4) solvents. Each cleavage reactions containing 50 μg of eluted affinity purified His-tagged SNB-1 and 3 μg of eluted affinity purified GST fusion proteins diluted in acidic or neutral solvents were incubated at room temperature for 24 hours. After incubation, a 10 μL aliquot of the reaction was used for SDS-PAGE analysis and the remaining solution was used in a gradient elution High Pressure Liquid Chromatography (HPLC). The HPLC analyses were performed on the 1100 Series HPLC (Agilent Technology) using an Eclipse C18 Plus column (3.5μm, 4.5 x 150 mm, Agilent technology). The mobile phase consisted of a mixture of 0.04% formic acid in water (A), acetonitrile (B), or methanol (C) was delivered at a flow rate of 1 mL/min. Step-wise gradients of A and B were simultaneously run in C, which produced linear gradients increasing from 4 to 6% of A and from 0 to 30% of B over 10 min. Afterward, proportions of A were kept constant at 6%, and B was rapidly increased to 90% over 2 min. An equilibrium phase of 2.5 min was allowed between samples [13].

2.7 Statistics:

All data were graphically presented as mean ± SEM. In the case of single mean comparisons, data were analyzed by two-tailed unpaired Student's t-tests. P-values < 0.05 were considered statistically significant.

3. Results

3.1 Analysis of RIC-4A and RIC-4B binding to VSM-1:

To better understand the function of VSM-1 in relation to SNARE proteins' regulation, we began investigating whether VSM-1 binds to neuronal specific SNAREs using *in vitro* binding assays. These experiments were performed by first affinity purifying His-tagged t-SNARE proteins RIC-4A and RIC-4B from *E.coli* cells containing plasmids ah126 and 127 respectively (Figure 1). Next, GST fusion proteins for full length VSM-1 (FL VSM-1), delta N terminus VSM-1 (ΔNT VSM-1), and GST (control) were also affinity purified from *E.coli* cells containing plasmids ah103, ah115 and pGEX 4T1 respectively (Figure 1). Next, 26-28 μg of GST fusion proteins bound to sepharose beads were incubated with varying amounts of eluted His-tagged RIC-4A and RIC-4B for 24 hours at 4°C. Last, interacting partners were pulled-down by centrifugation and protein-protein interactions were accessed using SDS-PAGE.

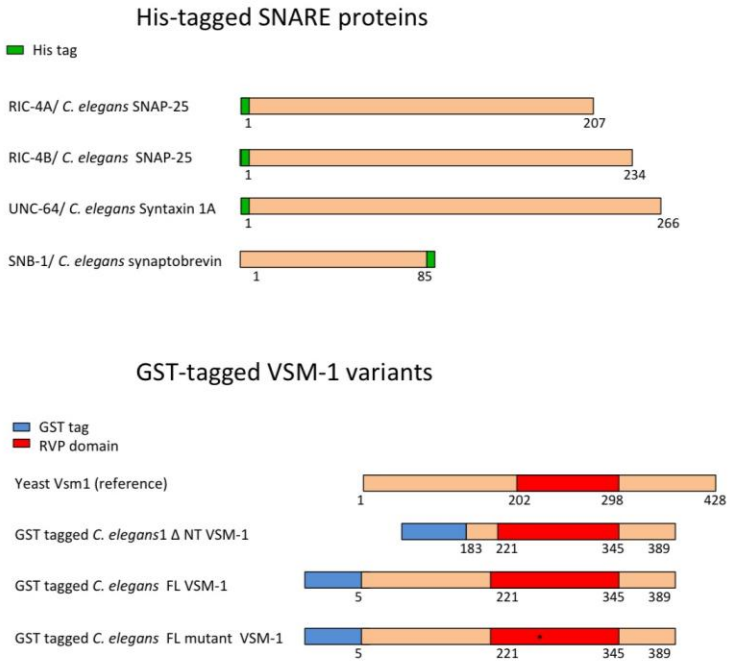

Figure 1: Schematic diagram of His-tagged SNARE proteins and GST-tagged VSM-1 variants used in this study. For each linear representation, left is N terminus, right is C terminus, green represents 6 X histidine tag (His tag), blue the Glutathione-S-transferase tag (GST tag), red the RetroViral Protease domain (RVP) and * denotes D261A point mutation. Numbers under each linear representation correspond to amino acid location in the polypeptide.

Analysis of three independent experiments resolved by SDS-PAGE and Coomassie Brilliant Blue R250 staining showed that *C. elegans* FL VSM-1 nor ΔNT VSM-1 co-precipitated with the t-SNAREs RIC-4A or RIC-4B (Figure 2A and B). Additionally, *in vitro* binding reactions containing 6000 ng, 9000 ng and 12000 ng of RIC-4A or RIC-4B showed the same banding pattern, indicating that increased concentration of the potential protein partner did not result in protein co-precipitation (Figure 2). Control tubes containing the GST tag with corresponding t-SNAREs RIC-4A or RIC-4B behaved similarly to the experimental tubes containing the GST tagged VSM-1 variants. Densitometry of regions of interest corresponding to potential RIC-4/B protein and GST fusion proteins (Beads) demonstrated that the percent ratio RIC-4/Beads was not statistically different when control GST tubes were compared to experimental tubes possessing FL VSM-1 or ΔNT VSM-1 (Figure 2). Together, these observations suggest that GST fused FL VSM-1 or ΔNT VSM-1 does not have a high affinity for binding RIC-4 isoforms *in vitro.*

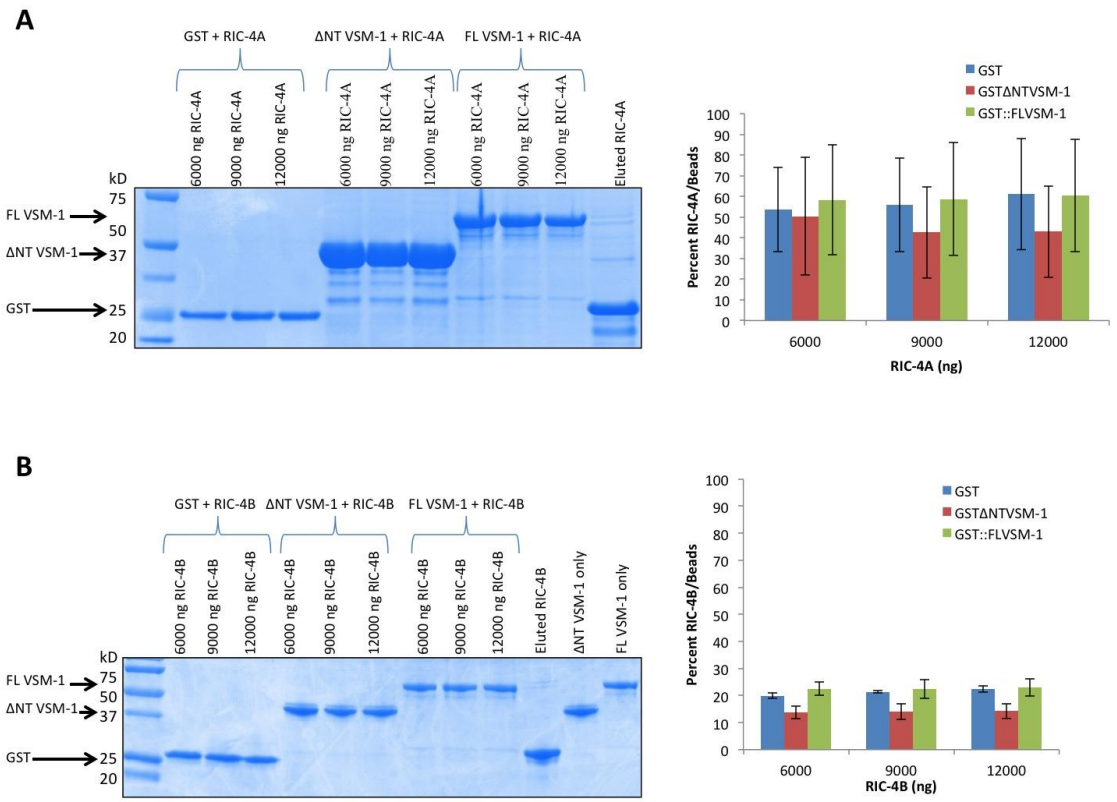

Figure 2: RIC-4A nor RIC-4B binds to *C. elegans* VSM-1 *in vitro*. GST-tagged VSM-1 proteins were incubated with 6000 ng, 9000 ng and 12000 ng each of RIC-4A (A) and RIC-4B (B) and analyzed via SDS-PAGE. A and B left panels show representative *in vitro* binding assays resolved using SDS-PAGE and Coomassie Brillant Blue stain. Densitometry of regions of interest performed from gels run for three independent experiments are graphed on right panels. Data plotted include percent average ratio of co-precipitated SNARE proteins to sepharose beads containing GST fusion protein (Beads) ± SEM. Student's t-tests analysis resulted in no statistical significant difference.

3.2 Examining SNB-1 and UNC-64 *in vitro* binding interactions to VSM-1:

To further investigate the role of VSM-1 in the regulation of exocytosis, we continued our SNARE binding analysis by examining the v-SNARE SNB-1 and the t-SNARE UNC-64. In this case, His-tagged UNC-64 and SNB-1 were affinity purified from *E. coli* bacterial cells expressing plasmids pRSETA and pH04sb61-62, respectively. Next, GST fusion bound proteins including ΔNT VSM-1, FL VSM-1, FL mutant VSM-1 (D261A) and GST were affinity purified and left attached to sepharose beads following the procedure described in material and methods. Sepharose beads containing proteins of interest were combined with increasing amounts of purified eluted His-tagged SNAREs ranging from 500-6000 ng and pull downs were examined after 24 hours incubation (Figure 3). Similarly to RIC-4 *in vitro* binding assays, increasing concentrations of eluted SNARE proteins did not promoted binding and co-precipitation with VSM-1 protein constructs. Both, the RVP domain of VSM-1 (ΔNT VSM-1) and the full length (FL VSM-1) failed to pull down SNB-1 and UNC-64 after 24h incubations (Figure 3). Protein band densitometry measurements show no significant difference between tubes containing GST (control), ΔNT VSM-1, FL VSM-1 nor FL mutant VSM-1 with respective t-SNARE UNC-64 or v-SNARE SNB-1 co-precipitation (Figure 3 graphs).
Finally, a last set of experiments containing 20 ug of affinity purified SNB-1 or UNC-64 and 20 ug GST, ΔNT VSM-1, FL VSM-1 and FL mutant VSM-1 beads also showed that beads coated with GST fusion VSM-1 variants do not pull down SNARE proteins UNC-64 and SNB-1 (Figure 4).

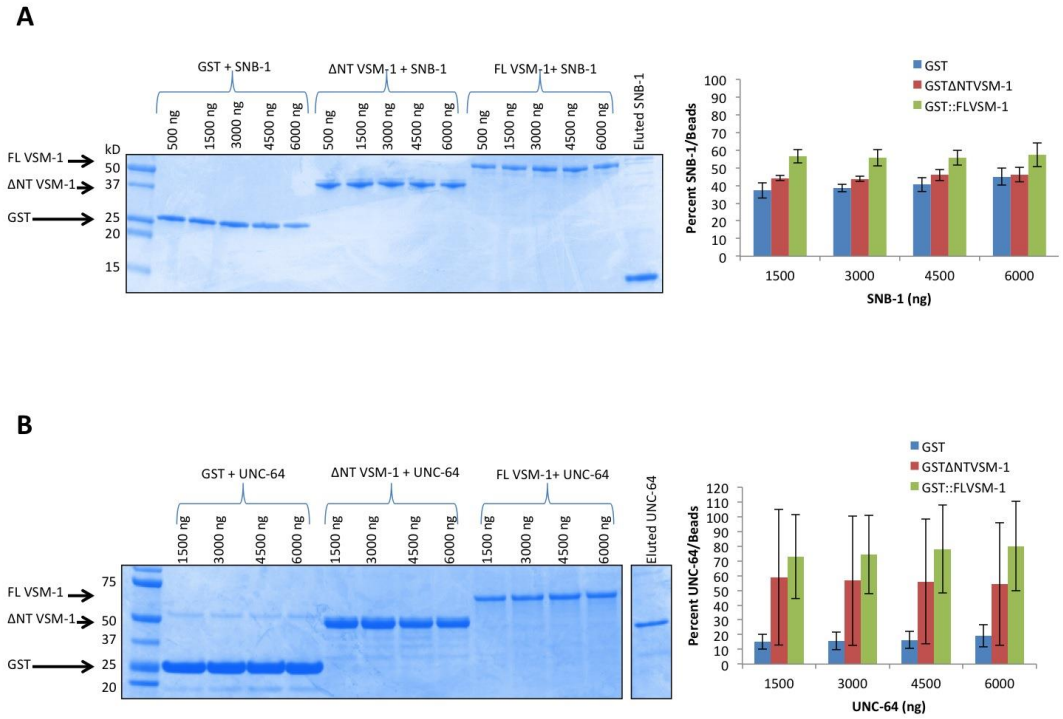

Figure 3: Pull down protein assays reveal no interaction between VSM-1 and SNB-1 nor UNC-64. A: Representative polyacrylamide gel image from pull down assays containing GST-tagged proteins and 500 ng to 6000 ng of SNB-1 (right). Coomassie Blue stained bands from three independent assays were quantified by determining region of interest and mean ± SEM are plotted (left). B: Representative image of similar pull down assay as A, where UNC-64 was applied to GST-tagged proteins (right). Quantification of three independent experiments is shown in the left panel. Plotted are mean ± SEM.

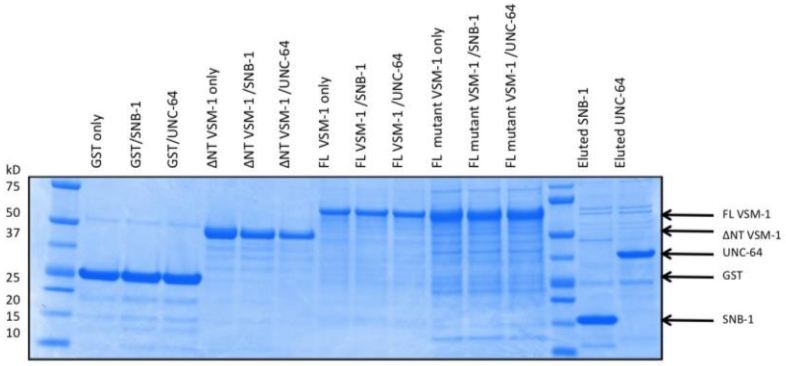

Figure 4: 20 ug of eluted SNB-1 and UNC-64 also show no specific binding to GST-tagged VSM-1 protein constructs. *In vitro* binding assays with glutathione sepharose beads bound to GST, ΔNT VSM-1, FL VSM-1 and FL mutant VSM-1 (D261A) and 20ug of SNB-1 or UNC-64 did not result in specific co-precipitation of VSM-1 and SNAREs.

Taken together, examinations of gels ran with pull down samples demonstrated that *C. elegans* VSM-1 does not co-precipitates with the t-SNARE UNC-64 nor v-SNARE SNB-1 under *in vitro* conditions. Thus, these results suggest that *in vitro* conditions employed here does not promote *C. elegans* VSM-1 interaction with SNARE or VSM-1 role

in membrane trafficking and exocytosis may be independent from interaction with neuronal SNARE proteins SNB-1, UNC-64, RIC-4A and RIC-4B.

3.3 Cleavage analysis between v-SNARE protein SNB-1 and VSM-1:

Reports from Gerst and colleagues established that an intact RVP domain in the VSM-1 protein is essential for its regulatory role in membrane trafficking and secretion [11]. Moreover, VSM-1 amino acid sequence analysis shows that the *C. elegans* protein posses a similar RVP domain too. For instance, 3D analysis of predicted protein structures generated using the University of Chicago's Raptor X software shows that *C. elegans* (A), *S. cerevisaie* (B) and *H. sapiens* (C) RVP domains have two alpha helices (pink) and a set of beta pleated sheets (yellow) that fold in a barrel-type conformation (Figure 5). Consequently, based on these observations and extensive literature on the proteolytic properties of RVP domains, we hypothesized that *C. elegans* VSM-1 inhibitory role in release may encompass proteolysis of the v-SNARE SNB-1, a phenomenon also seen with tetanus toxin [14-16]. To test this hypothesis and assess whether the full length VSM-1 with a wild-type RVP domain has proteolytic activity and cleaves SNB-1, we set up cleavage assays as described by Montecucco and colleagues [14]. Tubes containing 50 µg of eluted His-tagged SNB-1 and 3 µg of eluted affinity purified GST fusion proteins diluted in acidic or neutral solvents were incubated for 24 hours and cleavage was studied using SDS-PAGE. Results suggest that no proteolysis occurred, in other words, SNB-1 was not fragmented into smaller peptides when a protein containing a RVP domain was present in the cleavage assay (Figure 3 D). Likewise, investigations of cleavage using a more sensitive approach involving HPLC and two alternative pH environments indicated that the peak level/intensity of SNB-1 was unaltered in the presence of the putative protease/RVP domain (Figure 5 E). Peaks shown in reactions at pH 7.4 and FL VSM-1, or SNB-1 were also detected from cleavage reaction with both FL VSM-1 and SNB-1. No additional peaks denoting hydrolysis were detected. Hence, both SDS-PAGE and chromatography results did not report fragmentation of SNB-1 (Figure 3 D-E).

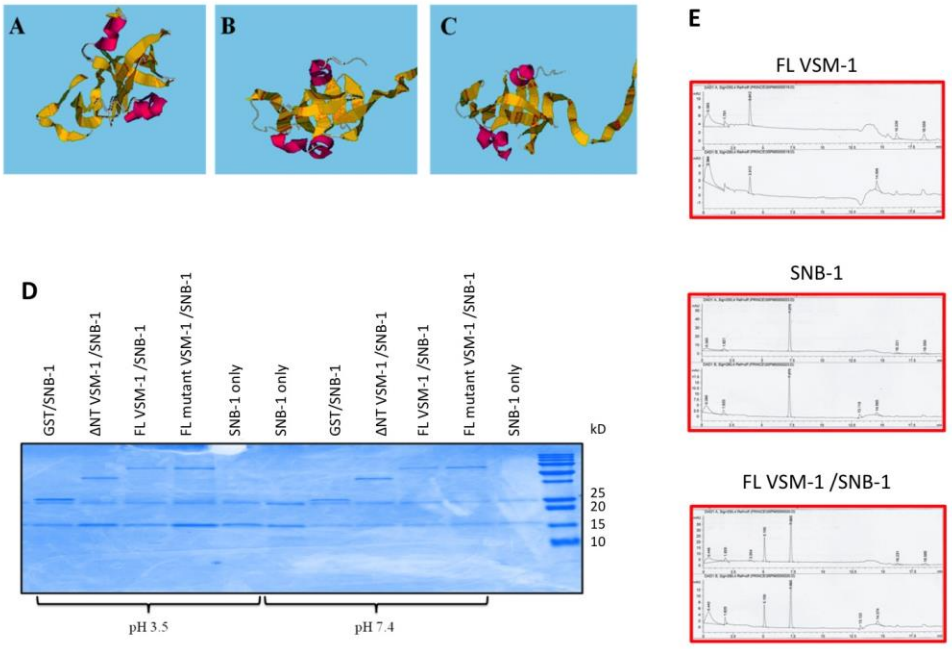

Figure 5: VSM-1 does not cleave SNB-1. Schematic of predicted 3-D protein structure for the VSM-1 RVP domain of *C. elegans* (A), *S. cerevisaie* (B) and *H. sapiens* (C) homologs produced using Raptor X software (U of Chicago). N terminus is top, C terminus right, alpha helices pink and beta pleated sheets yellow. D: SDS-PAGE Coomassie Blue stained gel representative image of three independent cleavage experiments. E: Cleavage experiments were also examined using HPLC. Chromatograms for samples containing full length VSM-1 fused to GST, SNB-1 and a combination of both are shown in E.

4. Discussion

Molecular signals are transmitted from one cell to another via a process known as exocytosis [17]. During exocytosis v-SNARE synaptobrevin/SNB-1 interacts with two t-SNARES syntaxin/UNC-64 and SNAP-25/RIC-4 to form a trimeric complex [18,19]. Assembly of SNARE complexes brings presynaptic vesicles into close proximity with the plasma membrane thus membrane fusion and exocytosis occur [2,20]. Previous research showed that VSM-1, a novel SNARE binding protein, has the potential of negatively regulating membrane fusion and secretion in yeast [10]. Specifically, the yeast homolog of VSM-1 was found to bind Snc1 and Snc2, two v-SNARE proteins important for vesicle to plasma membrane fusion [10]. Additional research from the same group also demonstrated that VSM-1 binds to the t-SNARE Sso1 displacing and competing with the t-SNARE Sec-9 [12]. Based on these findings, Gerst and colleagues postulated that VSM-1 is an inhibitory protein that binds to monomeric SNAREs and prevents the formation of fusogenic SNARE complexes [12]. Preliminary studies from our laboratory established that lack of VSM-1 in *C. elegans* results in enhanced ACh release as measured indirectly by increased aldicarb sensitivity [21]. Thus, to better determine the role of VSM-1 in neurons and neurotransmitter release, we began a biochemical study where protein-protein interactions were analyzed. Results obtained by our group show that in contrast to our hypothesized working model derived from the yeast Vsm1 findings, *C. elegans* VSM-1 does not co-precipitates with SNARE proteins SNB-1, UNC-64, RIC-4A nor RIC-4B. These results can be interpreted as if *in vitro* conditions did not promoted interaction of VSM-1 with neuronal SNAREs, or the interaction is transitory. Alternatively, failure to co-precipitate SNARE with VSM-1 protein constructs could be an indirect result of introducing GST, a large protein tag that may interfere with VSM-1 native protein folding and 3 dimensional shape. Moreover, *in vitro* conditions, buffer composition and pH may also play a role in reduced affinity or transient interactions. Additional test using endogenous VSM-1 and co-immunoprecipitation studies would shed light toward better interpreting these negative results. As for the information presented here, we postulate that although reports show that VSM-1 binds to SNAREs and regulates constitutive exocytosis in yeast; *C. elegans* VSM-1 does not seem to interact with neuronal SNAREs responsible for controlling Ca^{2+} regulated exocytosis via SNARE complex formation [22].

Alternatively to the VSM-1-SNAREs binding and competition model proposed by Gerst and colleagues [12], we investigated whether VSM-1 RVP domain mediates the cleavage of the v-SNARE SNB-1. Studies using the yeast homolog of VSM-1 show that an intact RVP domain is required for the inhibition of secretion [11]. Thus, based on the strong conservation of RVP domains found in yeast, human and *C. elegans* VSM-1, we tested proteolysis *in vitro*. A mixture of VSM-1 and SNB-1 showed no fragmentation of SNB-1 as assessed via SDS-PAGE and HPLC. Similarly to *in vitro* binding assays, we cannot rule out that the introduction of the GST tag could be the cause of no catalytic activity and proteolysis. The data resolved in gels and chromatograms using full length VSM-1 with a wild-type RVP domain is identical to a mutant version of full length VSM-1 where the RVP domain has been inactivated by replacing D261A.

To conclude, *in vitro* binding and cleavage assays reported here suggest that *C. elegans* VSM-1 does not co-precipitates with neuronal SNAREs UNC-64, SNB-1 and RIC-4A/B. Moreover, SNB-1 is not cleaved by VSM-1 protease *in vitro*. Thus findings of VSM-1 regulating constitutive exocytosis in yeast were not found to explain the negative regulation of exocytosis in the nematode calcium-mediated release. Instead, VSM-1 seems to inhibit exocytosis by some other means or binding properties of VSM-1 should be addressed with co-immunoprecipitation of endogenous VSM-1 and SNARES.

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6. Authorship Contributions

***** L.S., C.N., N.B. contribute equality in regards to the experimental component of this work. L.S. and A.H. wrote the manuscript. F.K. and A.P supervised the HPLC assays. A.H. supervised the rest of the project.

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