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The University of Southern Mississippi

The Subcloning and Expression of Munc18a in Escherichia coli for Antibody Production and Analysis in Mast Cell Degranulation Reactions

by

Brandi Goble

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirement for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2018

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Abstract

Mast cells are secretory cells responsible for fighting off infection through the early recognition of pathogens. This process is completed through the secretion of proinflammatory mediators that are stored in secretory granules within the cytoplasm of the cell. The degranulation secretion process relies on regulated fusion of secretory granules to the cell membrane via membrane-bound SNARE proteins that bridges the two opposed membranes. The intricate regulation of SNARE-mediated mast cell degranulation is not well understood. However, Sec1/Munc18 (SM) proteins, specifically the Munc18 isoforms, are known to play a critical role in the process (Brochetta, et. al., 2014). The Xu lab has recently demonstrated that Munc18a is phosphorylated in activated RBL-2H3 cells (a tumor analog of mucosal mast cells), and current research seeks to determine the function and physiological relevance of Munc18a phosphorylation. To achieve this, we are in the process of generating Munc18a-specific antibodies that would be used to specifically isolate Munc18a from resting and activated RBL-2H3 cell. Munc18a proteins will then be subject to mass spectrometry to identify novel phosphorylation sites. This project seeks to generate an epitope of Munc18a that will be used in this specific antibody production. This is being carried out by the amplification and isolation of a segment of the gene encoding Munc18a, subcloning it into bacterial expression vector pMBP-parallel, and expressing it in *E. coli* bacterium. In collaboration with others in the Xu lab, the isolated Munc18a cDNA will be used to raise antibodies with high specificity and sensitivity for Munc18a. I expect my research product to be essential for generating fresh new insights into the importance of Munc18a phosphorylation in mast cell degranulation.

Keywords: mast cells, Munc18a, proteins, antibody, *E. coli*, degranulation

Acknowledgements

I would like to begin by thanking Dr. Hao Xu for letting me be a part of his research lab while working on my research project and for teaching me the reasoning behind each of the steps that went into my completing my project. Dr. Xu pushed me to always do my best, work independently, and fully understand all aspects of my research. I learned to present my research in a scientific manner and be able to truly explain the concepts to others. I would also like to thank Cheri Voth for her guidance during each step of my research project and Pratikshya Adhikari for her assistance when Cheri was unavailable. They were both always there to offer their guidance, explain concepts, and answer any questions I had about my research process. Next, I would like to thank the University of Southern Mississippi Honors College for the opportunity to complete an undergraduate thesis and holding me accountable academically during my undergraduate career. Throughout my research process, I have become a better scientist and learned to fully appreciate the research process. I would also like to thank Mississippi INBRE for the use of their imaging facility. Lastly, I would like to thank the National Institute of Allergy and Infectious Diseases as well as the National Institute of Health for funding the research performed in Dr. Xu's lab.

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List of Abbreviations

SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
VAMP	Vesicle associated membrane protein
Munc18	*Mammalian uncoordinated18*
DNA	Deoxyribonucleic acid
PKC	Protein Kinase C
Ser-313	Serine-313

Chapter 1: Introduction

Mast cells are secretory cells found in connective tissues (e.g., respiratory tract, intestines and stomach lining) and are responsible for fighting off infection through the early recognition of pathogens. Mast cells accomplish this by secreting inflammatory mediators, such as histamine and proteases, which are stored in secretory granules within the cytoplasm of the cell. The secretion of these mediators, referred to as mast cell degranulation, could lead to allergic inflammation and anaphylaxis when mis-regulated. Degranulation relies on regulated fusion of secretory granules to the cell membrane (Lorentz et al, 2012). It is important to study how the degranulation process is regulated, because if the regulation process can be controlled, further studies can be done to positively affect the immune system. Membrane fusion, the conserved process by which mast cell degranulation can take place, is essential to intercellular trafficking. Membrane fusion begins as the transport vesicle approaches the target membrane, allowing for a hemi fusion of the outer lipid bilayers of each respective membrane (Figure 1). The fusion between vesicle and target membranes is catalyzed by specific proteins and lipids. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) are integral or peripheral proteins crucial to membrane fusion by forming a trans-complex that impose stress to the lipid bilayers (Figure 2).

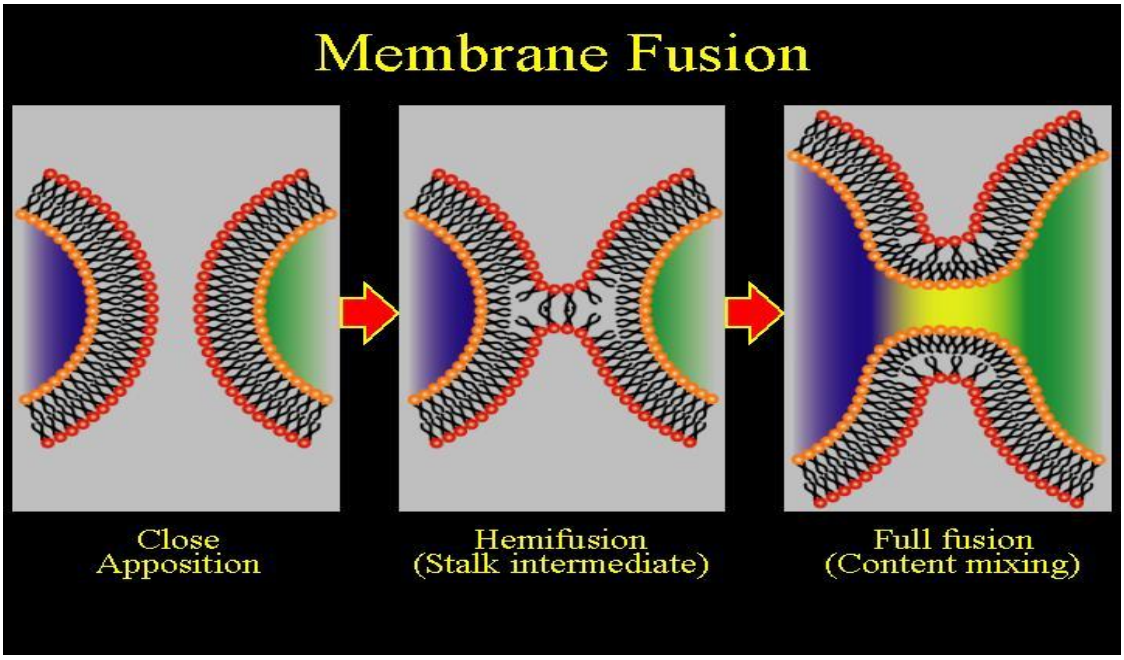


Figure 1: (A)The vesicle approaches the target membrane. (B) The outer regions of the lipid bilayer form a hemi-fusion attachment. (C) The inner bilayers combine, showing full fusion to take place. This allows for the translocation of secretory granules.

The SNARE complex that bridges two apposed membranes is formed by four helixes: three t-SNAREs and one v-SNARE coiled together (Alpadi et al, 2012). This family of SNARE proteins is made up of syntaxin, synaptobrevin/VAMP (vesicle associated membrane protein), and SNAP 25-like protein. VAMPs are found on the transport vesicle membrane and are classified as v-SNAREs. Syntaxin and SNAP 25-like proteins are found on the target cell membrane, and they are classified as t-SNAREs. Fusion of the vesicle and target lipid membranes relies on the formation of the trans-SNARE complex, made up of these SNARE proteins (Peijler and Wernersson, 2014). Although SNARE- mediated fusion has been the center of many research experiments,

the regulation of their assembly and disassembly is still unclear (Jorgensen and Palfreyman, 2008). However, we do know that interactions between different regulatory proteins and the SNARE complexes take part in the steps to regulate mast cell granule fusion. Three cytoplasmic proteins that interact with the SNARE complex, Munc18a, Munc18b, and Munc18c, make up the *mammalian uncoordinated18* (Munc18) family (Lorentz et al, 2012). The Munc18 proteins bind to the SNARE proteins and are thought to be required for all regulated exocytosis (e.g., neurotransmission, hormone secretion and mast cell degranulation).

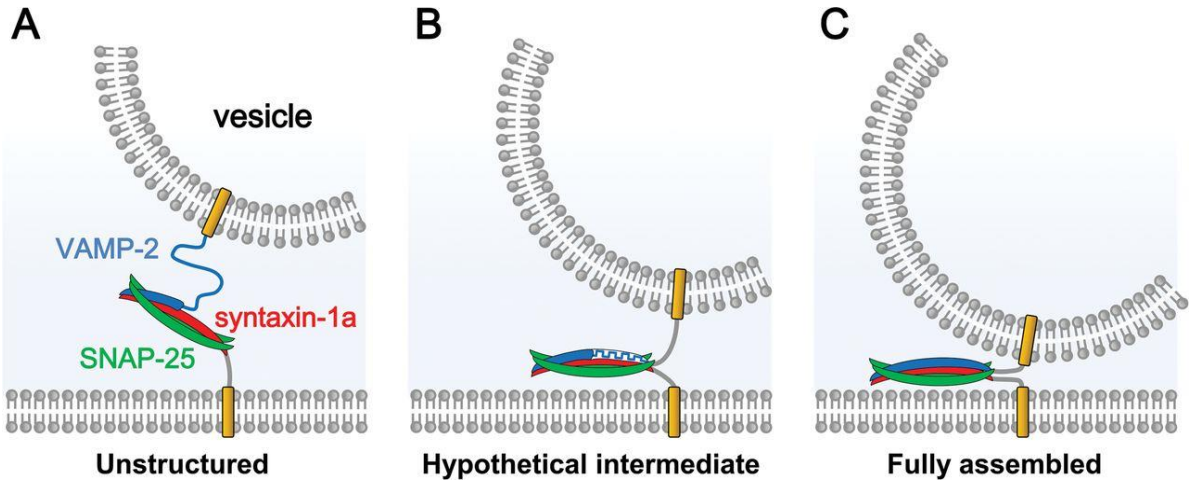


Figure 2: Membrane fusion via SNAREs. (A) shows the interaction between VAMP-2, syntaxin-1a, and SNAP 25 once the vesicle has moved close enough to the target membrane. (B) shows the formation of the trans-SNARE complex as the protein helices coil together, strengthening the bond. (C) shows the fully assembled SNARE complex. This allows for hemi fusion of the two membranes to begin, followed by full fusion (Jorgensen and Palfreyman, 2008).

Of the three Munc18 isoforms, recent studies have shown that Munc18a plays a role in both mast cell degranulation as well as neurotransmitter release in neuronal cells (Dulubova et. al., 2007). Munc18a has many roles in the regulation of SNARE-mediated membrane fusion. Munc18a's role in mast cell degranulation and neurotransmitter release relies on its interaction with SNARE proteins, syntaxin-1 and synaptobrevin. The C-terminus of synaptobrevin has a Munc18a binding site, allowing it to interact with the SNARE protein near the site of membrane fusion. The Munc18a protein also interacts with the N-terminus of syntaxin-1 to aid in facilitation of SNARE-mediated fusion by inducing lipid mixing (Xu et. al., 2010). The N-pocket of the D1 domain of Munc18a is what allows it to interact with the N-peptide of syntaxin-1 (Arnold et. al., 2017). Recent studies show that post-translational modification of Munc18a could play a role in how it can interact with these SNAREs to facilitate membrane fusion. For example, the phosphorylation of Munc18a at Ser-313 by Protein Kinase C is related to controlling exocytosis (Barclay et. al., 2003). Despite everything that we already know about the roles that Munc18a plays SNARE-mediated membrane fusion, there is still much to be learned about how exactly the Munc18a isoform specifically interacts with the different combinations of SNAREs that make up the trans-SNARE complex. We wanted to create the Munc18a epitope for antibody production so that we could obtain an endogenous Munc18a protein sample. Creating our own Munc18a-specific antibody instead of using a commercial antibody will ensure that only the Munc18a isoform binds to it, as well as cutting down on the expense of purchasing a specific antibody.

The goal for this research was to produce a successful gene construct for an epitope of Munc18a near the C-terminus that can be used in Munc18a- specific antibody

production for immunoprecipitation studies. The C-terminus was chosen as the site for our epitope because this area shows the most variation among the three Munc18 isoforms. While producing this gene construct for Munc18a is the first step in being able to produce Munc18a- specific antibodies, it is an extremely important step because it provides a base for which future studies in the Xu lab will build upon. This allows the antibody that will later be produced using our Munc18a protein a higher specificity. Production of this antibody will be completed by others in the Xu lab by injecting the Munc18a peptide into New Zealand White rabbits and allowing it to illicit an immune response (Figure 3). The rabbit's B-cells will produce antibodies against the peptide, that will serve as the antigen. The serum will then be harvested from the rabbit and affinity purified ("Practical Applications of Monoclonal and Polyclonal Antibodies").

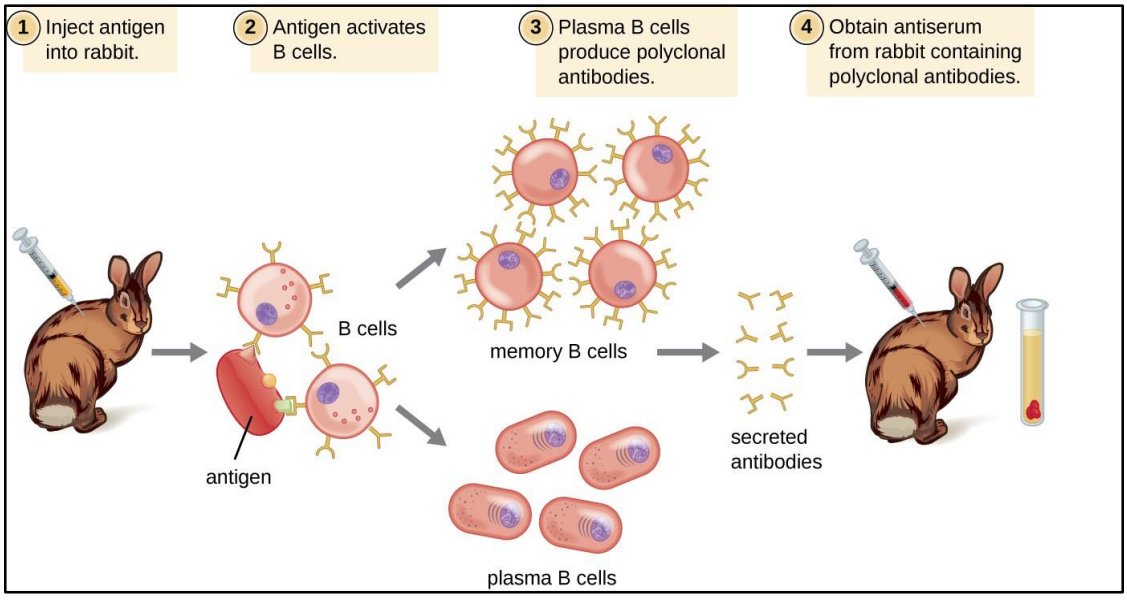
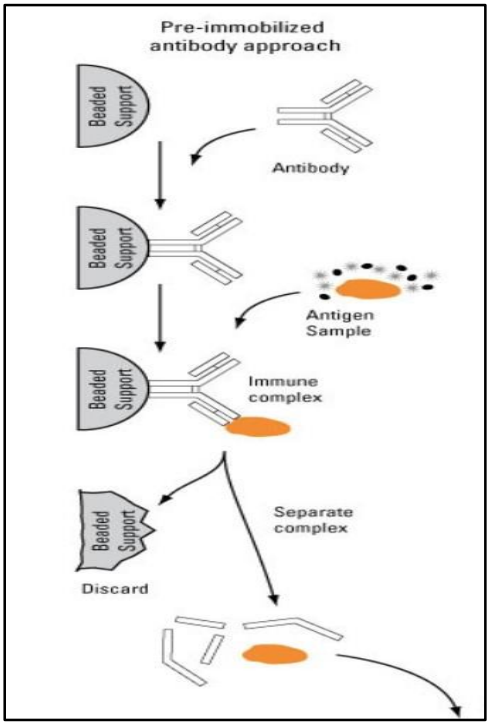


Figure 3: Antibody production process in rabbits.

The purified antibody will allow the Xu lab to isolate the endogenous Munc18a protein produced by RBL-2H3 cells through immunoprecipitation to be analyzed in future mast cell degranulation reactions ("Practical Applications of Monoclonal and Polyclonal Antibodies"). Immunoprecipitation will be performed in future studies using the obtained antibody for the desired Munc18a epitope near the C-terminus. The antibody pre-immobilized onto beads will bind with Munc18a from RBL-2H3 cell lysates in both resting and activated states. During incubation, the cell lysate will agitate and allow for the binding of the antibody and Munc18a, forming an immune complex. The complex will then be collected and separated from the beads for further analysis of the target antigen, Munc18a.



F**igure 4:** Immunoprecipitation will be performed using the obtained antibody for the desired Munc18a epitope near the C-terminus. The Munc18a will serve as the antigen and bind to the previously obtained antibody.

Chapter 2: Materials and Methods

Polymerase Chain Reaction

The first step in this process was to design and obtain primers for the Munc18a epitope sequence and amplify the cDNA by performing PCR. To begin PCR, 1uL (100ng/uL) of the plasmid DNA obtained for the Munc18a, 40uL of Millipore water, 1uL of 10 uM NcoI primer, 1uL of 10uM EcoRI primer, 5uL of 10x Pfu buffer, 1uL of 10mM dNTPs (New England Biolabs), and 1uL Pfu polymerase (G Biosciences part #) was placed in a PCR tube. PCR was then run according to the following times and temperatures to amplify the nucleic acids: 94°C/5min (1 cycle), 94°C/30s, 55°C/30s, 72°C/45s (5 cycles), 94°C/30s, 60°C/30s, 72°C/45s (25 cycles), 72°C/10min (1 cycle) then 4°C/∞. After completing PCR, the PCR product was analyzed by electrophoresis using 1% agarose gel. 10uL of each product was mixed with 2uL of gel red fluorescent dye to visualize the DNA and placed in its assigned compartment. It was run next to 6uL of 1kB DNA ladder with 2uL of gel red added for a total of 8uL. The remaining 40uL of each product was placed in the -20°C freezer for later use in the lab. The gel was set under UV light and a picture of the results was taken and saved in the computer files. After completing purification, Nano drop readings were taken to observe the concentration of the DNA (ng/uL) present. The pMBP-parallel vector was isolated

using a QIAprep Spin Miniprep kit per respective protocol before restriction and digestion began.

Restriction Digestion/Ligation

To begin restriction digestion, we mixed the 29uL of PCR products (Munc18a epitope) with 15uL of sterile millipore water. This was followed by adding 5uL of 10x NEB CutSmart buffer (Biolabs), and 0.5uL of each restriction enzyme, NcoI and EcoRI (Biolabs). This process was repeated with 40 uL of vector pMBP-parallel DNA, except without the H_2O. The mixtures were incubated at 37°C for 3 hours. Next, the vector was inactivated by incubating at 65°C for 20 minutes, then placed on ice for 2 minutes. 1uL of alkaline phosphatase, used to dephosphorylate the 5' and 3' ends of the DNA, was added to the vector only sequence and incubated at 37°C for 1 hour. The vector was then heat inactivated at 65°C for 20 minutes. This process allowed the vector plasmid, pMBP-parallel, to open and create sticky ends on both the vector and the Munc18a epitope in preparation for the recombination of the DNA (Figure 5).

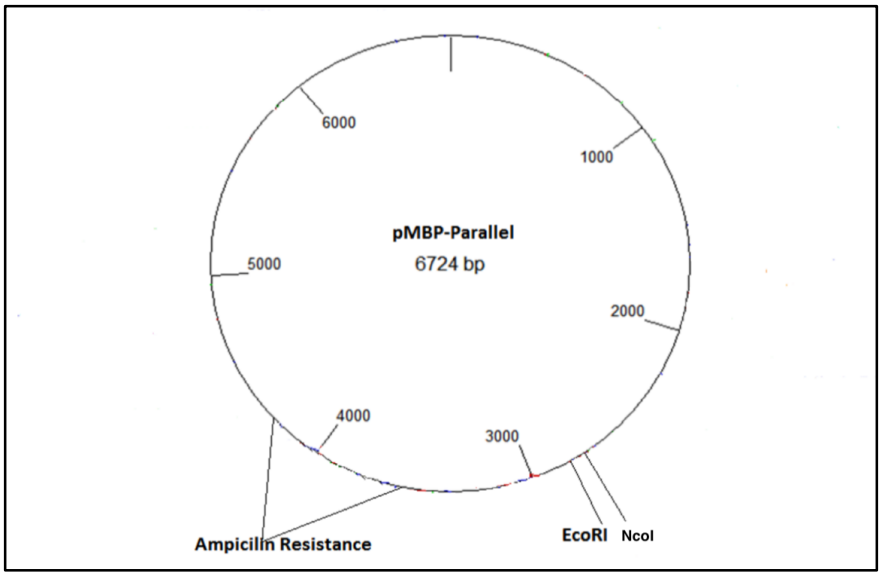


Figure 5: This figure shows where restriction enzymes, EcoRI and NcoI cut vector, pMBP-parallel, to allow for ligation of the insert. These restriction enzymes were chosen because they cut the vector once and do not cut the insert. NcoI and EcoRI cut 11bp from the pMBP-parallel vector. The ampicillin resistance was selected to ensure that, following restriction digestion, no contaminates survived the transformation step.

Following restriction digestion of both the insert and the vector, both restriction digestion products were run on a 0.7% agarose gel at 80V for 1 hour using gel red to fluoresce. The vector was run next to a 6λ DNA ladder. The insert was run next to a 1kB DNA ladder. A gel extraction of the restriction digestion products was performed next using the QIAprep Gel Extraction Kit protocol. The restriction digestion vector product was 0.26g and the insert product was 0.169g. To begin ligation, ligation buffer, T4 DNA ligase, insert DNA, and vector DNA was added to labeled microcentrifuge tubes (Table 1). The tubes were incubated overnight at 17°C. This allowed for the Munc18a cDNA to attach to the selected vector plasmid at the sticky ends.

Table 1- Ligation protocol.

	Munc18a (insert) + pMBP-parallel (vector)	**pMBP-parallel (vector/ control)**
T4 Ligase	1uL	1uL
10x Buffer	1uL	1uL
pMBP-parallel vector	3uL	3uL
Munc18a insert	5uL	0uL
Millipore water	0uL	5uL

Transformation of Novablue

Once the ligation of the Munc18a with the pMBP-parallel vector was complete, the next step was to transform competent *E. coli* to select for the engineered construct (vector plus insert). To begin this process, the competent cells were thawed on ice. The cells were put into chilled 1.5ml micro-centrifuge tubes taken in 15uL increments. 1.5uL of each ligation product was mixed with the 15uL cells in tubes by flicking the tube. After the tubes are completely mixed, they were placed on ice for 30 minutes, then heat shocked for 45 seconds at 42°C in a heating block, then put back in the ice immediately for 10 more minutes. 75uL of S0C media was added to each tube and incubated at 37°C for 1 hour. After the 1-hour incubation was complete, each mixture was transferred to LB/Ampicillin (100ug/mL) agar plates with 5-7 sterile glass beads. To spread the cells out evenly on the plates, the plates were rotated horizontally on the bench for 15 seconds.

Once the glass beads were removed, the plates were placed in the incubator for 20 minutes, then inverted overnight at 37°C.

Plasmid Isolation/Sequencing

Within the next year, further studies will be conducted in the Xu lab to attempt to confirm the Munc18a sequence through sequencing of the plasmids grown during transformation. Colonies grown on the plates overnight will be inoculated and isolated before being sent off for sequencing. Upon receiving the sequencing results, a nucleotide BLAST will be run on the NCBI website of our sequence results with the predicted sequence of our Munc18a. From this BLAST, the two sequences can be compared to determine if any error occurred or if the presence of Munc18a is confirmed.

Chapter 3: Results and Discussion

Munc18a rat cDNA was used to amplify an epitope near the C-terminus using PCR. PCR amplification was performed using custom primers. A 561bp construct was created using a forward and a reverse primer (Figure 6).

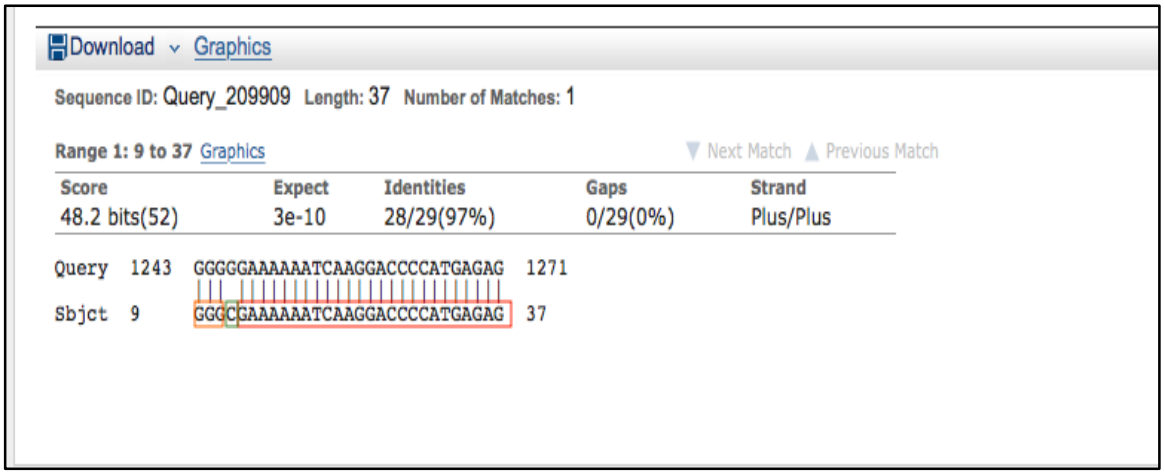


Figure 6: A nucleotide BLAST was performed showing the alignment between the Munc18a epitope sequence and the custom designed forward primer which includes an EcoRI restriction site. The nucleotides indicated by the red box depict epitope sequence of Munc18a. The green box depicts an additional cytosine that was added to the primer sequence to keep the sequence in reading frame after cloning into the expression vector. The orange box depicts where the restriction site begins.

Gel electrophoresis of the PCR product was carried out using 1.0 % agarose gel. A 1Kb DNA ladder was used to measure the size of the fragments present. 18x gel red was used to fluoresce samples. The epitope sequence of Munc18a near the C-terminus was successfully amplified using PCR (Figure 7). The PCR product was visually confirmed using the band located at the 0.6kB mark compared to the 1kB DNA ladder run next to it. The desired Munc18a epitope sequence is 561 bp, so a band was expected right above the 0.6kB mark where the arrow is indicating our sample was.

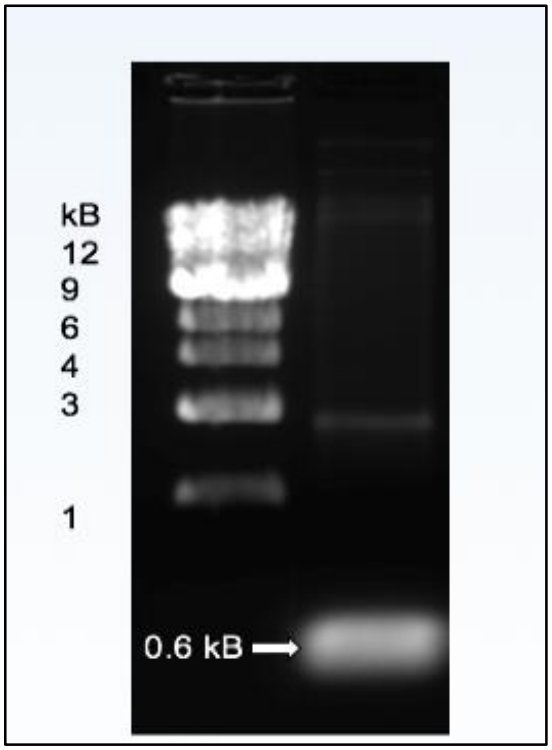


Figure 7: This figure shows a 1kB ladder in the left lane and PCR products in the right lane. The gel confirms that the desired 0.6kB insert was amplified using PCR, as indicated by the arrow.

Following PCR amplification, the PCR product was digested along with the pMBP-parallel vector using EcoRI and NcoI restriction enzymes. The restriction enzymes were used to cut 11bp from the vector and create sticky ends to prevent the plasmid from self-ligating. These cuts in the vector allowed for the Munc18a epitope sequence to be inserted into the vector. Restriction enzymes EcoRI (BioLabs Cat# 0331205) and NcoI (BioLabs Cat#0301209) were selected as both enzymes cut the vector plasmid once while not cutting the Munc18a cDNA. EcoRI was also chosen for its maintenance of reading frame. Restriction digestion was performed on partial Munc18a

cDNA (Figure 8) as well as the vector plasmid (Figure 9). Gel electrophoresis was performed using 0.7% agarose, followed by gel extraction of all products.

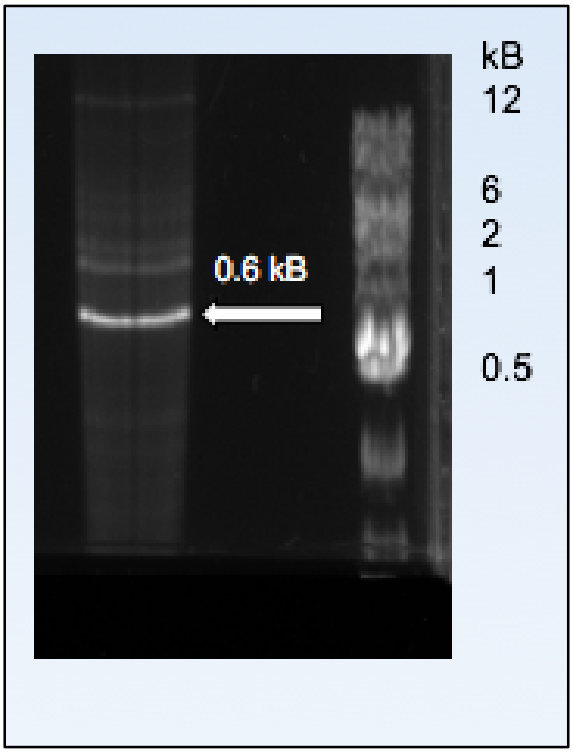


Figure 8: Restriction digestion of insert, Munc18a, was performed and run next to a 1kB DNA ladder. The insert is in the left lane, and indicated with the arrow 0.6kB. The right lane shows a 1kB ladder. This image can be compared to Figure 4 showing the undigested insert to confirm the presence of Munc18a.

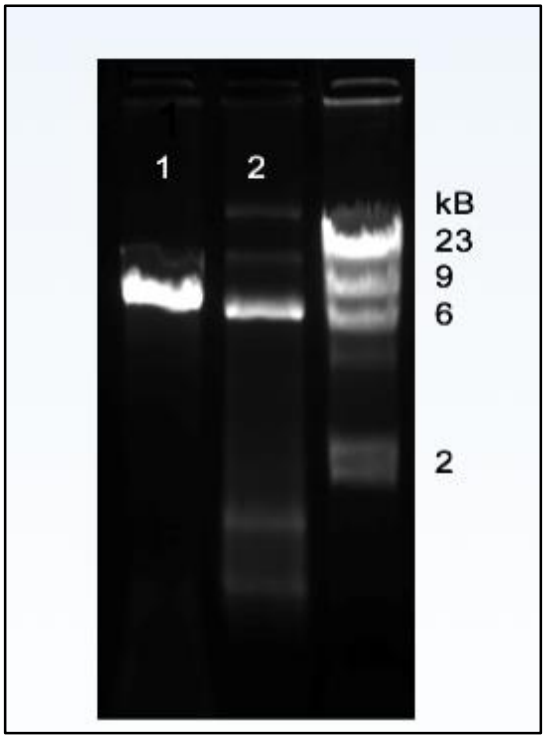


Figure 9: Restriction digestion of pMBP-parallel was performed and run next to a 6λ DNA ladder. Lane (1) shows the undigested vector and lane (2) shows the restriction digested vector. The 6λ DNA ladder was run in the far-right lane. The gel shows that the digested and undigested vector are present at around 6kb.

A Nano-drop reading was taken after a gel extraction of the restriction digestion products (Table 2) (Table 3). Approximately 1.8nm is a pure Nano-drop reading for 260/280 ratio, and approximately 2.0nm is pure for a 260/230 ratio. The restriction digestion of the PCR products has a 0.95nm Nano-drop reading for the 260/280 ratio, and a 0.01nm reading for the 260/230 ratio (Table 2). These lower Nano-drop readings indicate that contamination of a protein, phenol, or other reagent used in the DNA extraction is present. The restriction digestion of the vector has a 2.24nm reading for the

260/280 ratio, and 0.02nm reading for the 260/230 ratio (Table 3). The high 260/280 ratio for the pMBP-parallel vector could indicate that there is an RNA contamination.

	ng/uL	260/280	260/230
Munc18a	20	1.14	1.63

Table 2- Nano-drop readings after PCR purification. The 260/280 and 260/230 ratios show that there is a slight contamination. This could have occurred during preparation of the PCR mixture or during the purification of the PCR products.

	ng/uL	260/280	260/230
Munc18a	2.4	0.95	0.01
pMBP-parallel	10.4	2.24	0.02

Table 3- Nano-drop reading after gel extraction. The 260/280 and 260/230 ratios show that there is a possible protein, contamination, or organic compound contamination. This could have occurred the restriction digestion process or the gel extraction.

Following restriction digestion, ligation of the vector and insert was performed (Table 1). The recombinant plasmid containing the pMBP-parallel vector and Munc18a epitope sequence were then transformed into *E. coli* expression strain, Novablue. (Figure 10). This allowed for the plasmid to be transformed into the Novablue *E. coli* strain for expression. The plate on the left contains both the vector and insert. The plate on the right served as a control and contains only the vector mixed with millipore water (Figure 10).

We observed that 11 colonies grew on the ampicillin resistant growth medium on Plate 1. Plate 2, the control, showed no colony growth. Successful ligation of the vector and insert was confirmed by the colony growth on Plate 1 (Figure 10). Ligation of the vector and insert allowed colonies to grow because of the plasmid's selected marker for ampicillin resistance. Plate 2, showing no colony growth, confirmed that there was no presence of the Munc18a insert added. Although the pMBP-parallel vector itself contains an ampicillin resistance, without the insert added, the plasmid cannot be transformed.

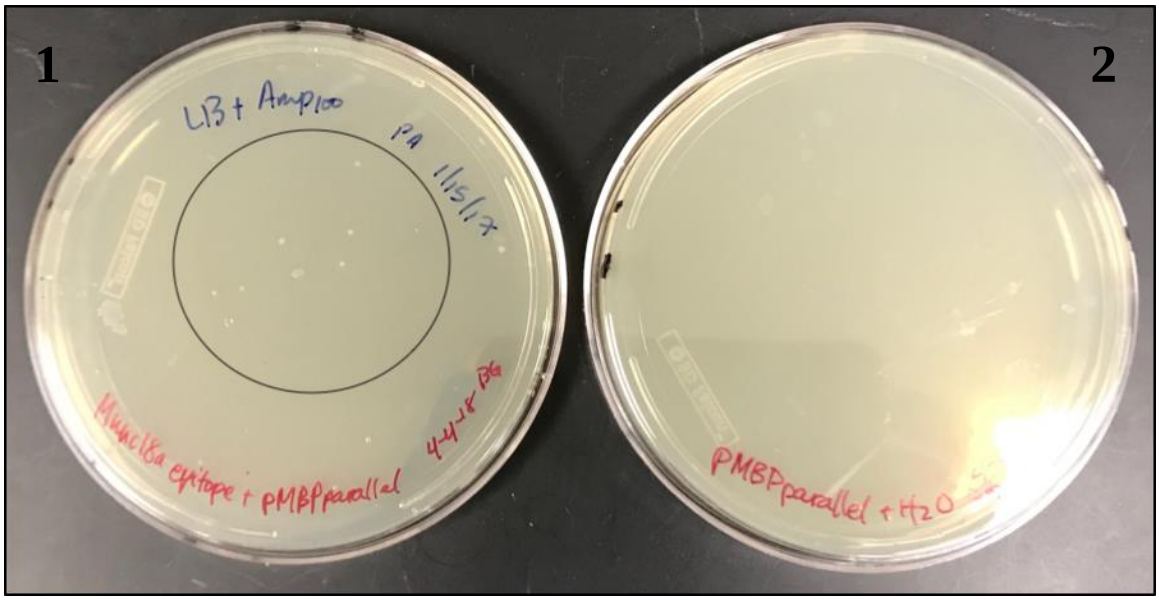


Figure 10: (1) Plate contains Munc18a epitope (insert) and pMBP-parallel (vector). Successful ligation and transformation into Novablue competent cells was confirmed by the growth of colonies (2) Plate only contains pMBP-parallel and millipore water to serve as a control. No colonies are seen.

Future studies conducted in the Xu lab will involve re-isolating the plasmids from competent Novablue cells and sending them to be sequenced through Eurofins. The sequencing results will be able to confirm the presence of Munc18a. Once the Munc18a sequence is confirmed, the cloning product will be sent for use in antibody production, followed by immunoprecipitation to obtain an endogenous protein. The endogenous Munc18a protein will allow us to further study the role that Munc18a plays in SNARE-mediated membrane fusion.

Although Munc18a is not the only protein that aids in SNARE-mediated fusion, this research is extremely important because the more we understand about how the SNARE-mediated fusion process is controlled, the more influence we have over manipulating when it occurs. Being able to control SNARE-mediated fusion and in turn, mast cell degranulation, could impact how physicians can treat allergies. As of now, physicians are only able to treat allergies with limited success. According to the American College of Allergy, Asthma, and Immunology (2016), over 50 million Americans are still affected by allergies, and allergic disease is the sixth leading chronic disease in the U.S. Being able to control the fusion process in mast cells can lead to better treatment options for allergies and possibly prevention altogether. This not only can positively improve a patient's quality of life, but it can also ease the financial burden of allergy treatment. In one year alone, the cost of allergy treatment in America is approximately 18 billion dollars (*American College of Allergy, Asthma, and Immunology*, 2016).

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