

Spring 5-2014

Subcloning and Expression of Complexin Isoforms Involved in Mast Cell Degranulation

Cameron Blake King
University of Southern Mississippi

Follow this and additional works at: https://aquila.usm.edu/honors_theses



Part of the [Cell and Developmental Biology Commons](#)

Recommended Citation

King, Cameron Blake, "Subcloning and Expression of Complexin Isoforms Involved in Mast Cell Degranulation" (2014). *Honors Theses*. 226.
https://aquila.usm.edu/honors_theses/226

This Honors College Thesis is brought to you for free and open access by the Honors College at The Aquila Digital Community. It has been accepted for inclusion in Honors Theses by an authorized administrator of The Aquila Digital Community. For more information, please contact Joshua.Cromwell@usm.edu.

The University of Southern Mississippi

Subcloning and Expression of Complexin Isoforms Involved in Mast Cell Degranulation

by

Cameron King

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

May 2014

Approved by

Hao Xu, Ph.D., Thesis Adviser
Assistant Professor of Biology

Shiao Wang, Ph.D., Interim Chair
Department of Biological Sciences

David R. Davies, Ph.D., Dean
Honors College

Abstract

Mast cells play an important role in the immune system by releasing chemicals such as chemokines and cytokines once they are stimulated. These products are released after stimulation by a process called mast cell degranulation. Mast cell degranulation is accomplished when vesicles containing the chemicals inside the mast cell fuse with the mast cell membrane via SNARE-mediated (Soluble NSF Attachment Protein Receptors) membrane fusion. This family of proteins consists of syntaxin, SNAP 25-like protein, and synaptobrevin/VAMP (Vesicle Associated Membrane Protein)(2). Complexin isoforms (complexin 1,2,3,and 4) have been known to regulate this system in a fashion that is still unclear. In order to study the mechanism in which these complexins regulate SNARE-mediated membrane fusion, each isoform was cloned and ligated to the pTYB12 vector to be expressed in *E. coli*. An induction process using IPTG was used in order to induce production of each isoform via the T7 promoter. In this experiment, we were able to clone all of the complexin isoforms, but only complexin 1 and 3 were successfully expressed.

Key words: Mast cell, SNARE, complexin, degranulation

Acknowledgements

First off I would like to thank Dr. Hao Xu for being willing to allow me to complete a research project in his lab and for taking time to teach me about many aspects of the research process. I really learned a lot about critically thinking and truly understanding every aspect of what you are doing from Dr. Xu. Also, I would like to thank Matthew Arnold for guiding me through every step of the research project, and Sushmitha Vijaya Kumar for her help when Matthew was not available. They were always willing to help and give guidance when it was needed. Without the help of these two grad students I would have had a much more difficult time obtaining the results I got. I also want to thank the University of Southern Mississippi Honors College for accepting me into their program and allowing me to complete an undergraduate thesis project. Through this process, I have learned a lot about myself and about important research techniques in the biological sciences. I also would like to acknowledge the USM Development Grant for Dr. Xu for funding the research in his lab.

Table of Contents

List of Tables.....	vii
List of Figures.....	viii
Introduction.....	1
Materials and Methods.....	6
Data and Results.....	12
Discussion.....	19
Literature Cited.....	21

List of Tables

Table 1: Primers used for PCR.....	7
Table 2: 10% SDS-PAGE Gels.....	11
Table 3: 5% stacking Layer.....	11
Table M1 - Nano-drop readings after PCR cleanup.....	13
Table M2 - Nano-drop reading after gel extraction/pTYB12 vector isolation.....	13
Table M3 - ligation set up.....	14

List of Figures

Figure 1 Membrane fusion via membrane lipids	1
Figure 2 Proposed model of membrane fusion via SNAREs	2
Figure 3 Complexin-SNARE complex in mammals	4
Figure 4 Possible model for complexin as a regulator	5
Figure 5 PCR Results.....	13
Figure 6 Complexin 1 sequencing results.....	14
Figure 7 Protein BLAST of complexin 1.....	15
Figure 8 Complexin 2 sequencing results.....	15
Figure 9 Protein BLAST of complexin 2.....	16
Figure 10 Complexin 3 sequencing results.....	17
Figure 11 SDS-PAGE results.....	17
Figure 12 PCR confirmation of complexin 4.....	18

Chapter 1: Introduction

Membrane fusion is critical in the secretion of biologically active factors such as hormones and neurotransmitters, as well as protein transport within the cell. It requires selected proteins and lipids to catalyze a series of steps that lead to the merger of two biological membranes (figure 1). Membrane fusion is catalyzed by a family of proteins known as SNARE proteins, which are conserved in all Eukaryotic systems. The proteins that make up this family are syntaxin, SNAP 25-like protein, and synaptobrevin/VAMP (vesicle associated membrane protein)(2). These SNAREs fall under two different groups: v-SNAREs (VAMPs), which are found on the transport vesicle and t-SNAREs (Syntaxins and SNAP 25-like), which are found on the target membrane (1,4). The core machinery for membrane fusion is the formation of a SNARE complex.

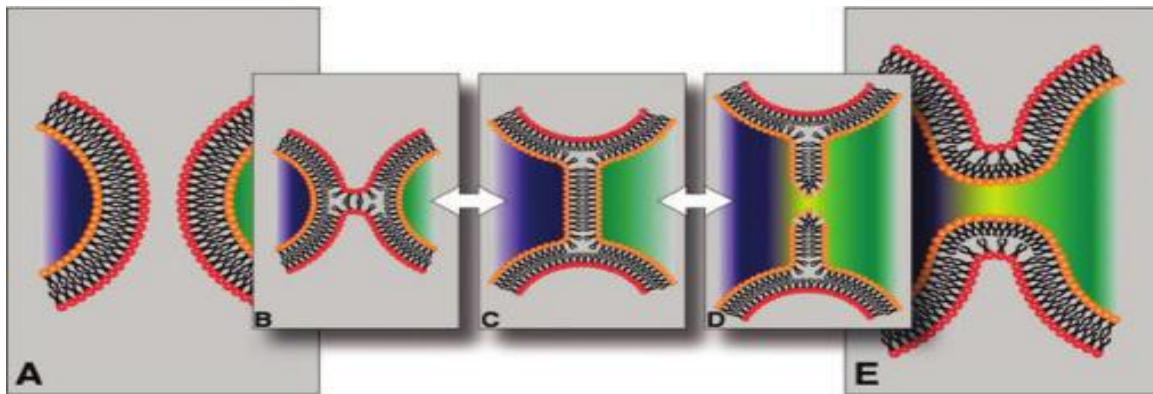


Figure 1: Membrane fusion via membrane lipids. As two vesicles approach each other (A), the outer regions (red) of both bi-layered membranes merge resulting in hemi-fusion (B). The inner layer of the bilayer then forms a stalk with only the inner membrane left to fuse (C). The two inner layers of the membrane (orange) then come in contact with each other (D). As fusion continues, the membranes from the two vesicles become one continuous membrane allowing for the exchange of content between vesicles. Image from (1)

A SNARE complex is formed when SNARE proteins from two opposing membranes interact with each other as shown in Figure 2, which then allows for the fusion of the two membranes. Although the general principles for fusion seem to be conserved from organism to organism, the regulation of specific SNARE-mediated fusion events in systems containing many combinations of SNARE proteins is less clear (1).

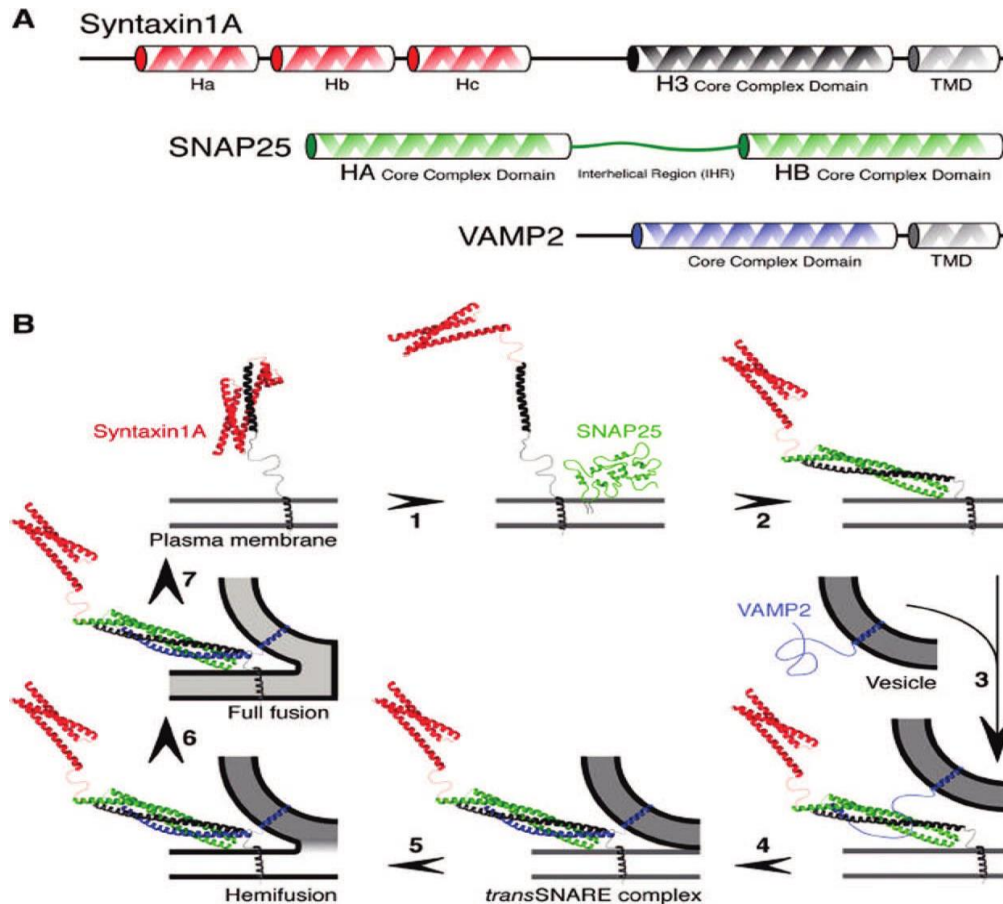


Figure 2: Proposed model of membrane fusion via SNAREs. A- represents proteins involved. B- step one involves the migration of Syntaxin 1A closer to SNAP 25. SNAP 25 then interacts with the H3 (black) domain of Syntain 1A. As the vesicle approaches the target membrane, VAMP 2 interacts with the SNAP 25-H3 domain complex. The interaction of these 3 proteins results in the formation of a *trans*SNARE complex. This is followed by hemi-fusion then full fusion of the two membranes. Image from (1)

Although all cells require membrane fusion, certain cells are more specialized in SNARE-mediated fusion events, i.e. mast cells. Mast cells are specialized secretory cells that respond to inflammatory signals with the release of a wide variety of products, stored in secretory granules, such as histamines, proteases, and cytokines/chemokines (3,4), which play important roles in the innate and adaptive immune system. The release of these products is referred to as mast cell degranulation. Once a mast cell is activated the degranulation process begins within a few seconds and is complete 5-10 seconds later (3). Degranulation results from a type 1 hypersensitivity reaction. This type of reaction occurs when an allergen-antibody complex (allergen that is bound to an IgE immunoglobulin) binds to the IgE Fc receptor on the surface of the mast cell. The binding of the Fc region of the allergen-antibody complex to the Fc receptors on the mast cell initiates various chemical reactions that lead to degranulation. Although mast cells are involved in these type 1 hypersensitivity reactions they can also promote or suppress inflammation. Mast cells and the chemokines/cytokines and other mediators they secrete activate antigen presenting cells, Langerhans cells, and dendritic cells for migration, which can cause tissue damage if the response is too strong (5). It is important to understand various ways in which mast cells degranulation can be regulated and how this degranulation can affect the immune system. Recent studies of mast cells have shown that the degranulation process relies on the same mechanism of SNARE-mediated membrane fusion in order to release the granule contents of the mast cells (4).

Many different proteins can regulate mast cell-granule fusion via interaction with SNARE complexes. Perhaps the most controversial SNARE regulators are the members of the complexin family (4). There are 4 isoforms in the complexin family: complexin 1,

complexin 2, complexin 3, and complexin 4 (1). Complexins were thought to be primarily restricted to the nervous system; however, they have been recently identified in testis, pancreatic beta cells, and other cells that perform regulated secretion such as mast cells. Complexin 2 has been found to be required for mast cell degranulation (4,8), but less is known about the roles of other isoforms in the process.

A model for the role of complexin in membrane fusion is demonstrated by the intricate interaction between complexin 1 and the neuronal SNAREs (Figure 4). When complexin 1 interacts with the SNARE complex, it does not directly bind to an individual SNARE protein. Instead, it interacts with a central α -helical domain within the assembled SNARE complex as shown in figure 3 (7). However, it is unclear whether other complexin isoforms regulate membrane fusion in a similar fashion. By cloning and expressing the complexin isoforms, we can begin to study their specific interactions with SNARE proteins.

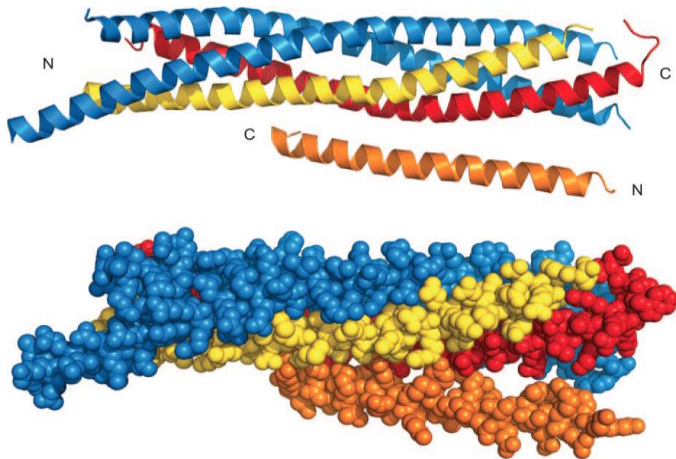


Figure 3 Complexin-SNARE complex in mammals. Blue- SNAP 25, Yellow- Syntaxin 1, Red- Synaptobrevin-2(VAMP2), Orange- complexin 1. Complexin 1 binds to the SNARE complex in the groove between syntaxin 1 and VAMP 2 in an antiparallel fashion with the C terminus of complexin lined up with the N terminus of the SNARE complex. Image from (7)

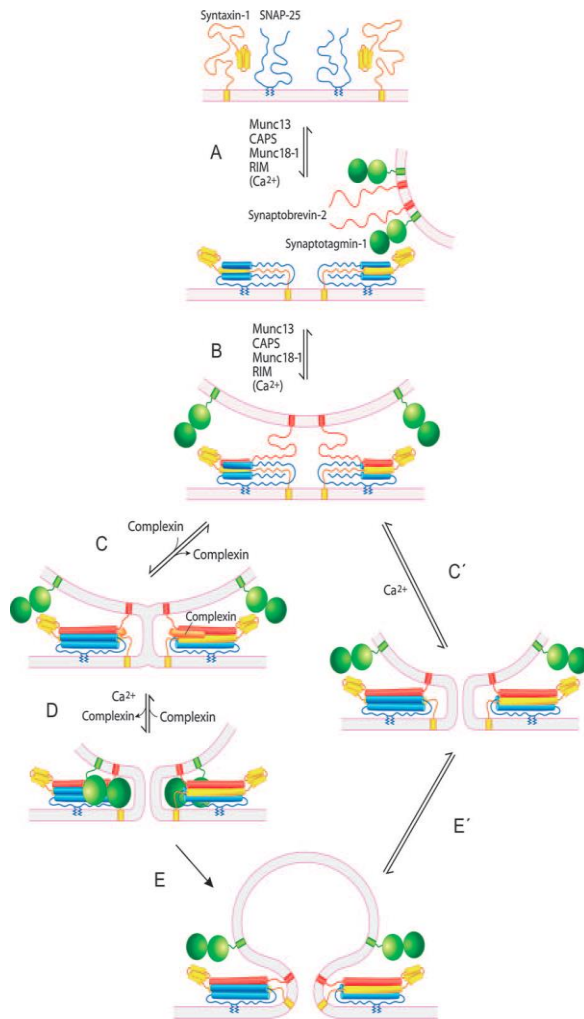


Figure 4: Possible model for complexin as a regulator. As the SNARE complex forms between the two membranes, complexin interacts with the SNARE complex inhibiting immediate fusion. Complexin is released from the SNARE complex upon calcium influx and interaction with synaptotagmin-1. Fusion occurs after the release of complexin (7).

Chapter 2: Materials and Methods

PCR

1uL (100ng/uL) of the plasmid DNA obtained for the complexin 1, 2, 3, and 4 isoforms were placed in its own PCR tube along with 40uL of HPLC (High performance Liquid Chromatography) water, 1uL of 10 uM Nde 1 primer specific for each isoform, 1uL of 10uM EcoR1 primer specific for each isoform, 5uL of 10x Pfu buffer, 1uL of 10mM dNTPs (New England Biolabs #N0447S), and 1uL Pfu polymerase (G Biosciences part #108P-A). The PCR was then run using this setup: 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 running the PCR, the PCR product was run on at 100V for 1 hour on a 1% agarose gel. 10uL of each product was mixed with 2uL of 6x loading dye and placed in its corresponding well. A Tridye100bp DNA ladder (#N3271S from New England BioLabs) was also used to help verify the product. The remaining 40uL of each product was placed in a -20°C freezer for later use. After the gel run, the gel was placed under UV light and a picture was taken and saved on the computer to record the results of the gel run. (Note: Complexin 1 and 2 were run with Rat templates and complexin 3 and 4 were run with Mouse templates due to availability and the fact that both rat and mouse complexin protein sequences are identical.)

Table 1 - Primers used for PCR

	Forward primer	Reverse complement primer
Complexin 1	ggatattCATATGGAGTTCGTGATG AAACAAG	cgGAATTCTTACTTCTTGAACATGTCC TG
Complexin 2	ggatattCATATGGACTTCGTCATG AAGC	cgGAATTCTTACTTCTTGAACATGTCC TG
Complexin 3	ggatattCATATGGCGTTCATGGTG AAGTCC	cgGAATTCACATGATATGGCACTTCTC
Complexin 4	ggatattCATATGGCTTTCCTTTGTG AAAAATATG	cgGAATTCACATCACAGAACAACACTTCT G
Sequencing primer HXO_C63	TTTGCACGTGAGTGCCGCGG	

I Used a Quigen PCR purification kit (50) Cat. No. 28104 to do PCR purification. The protocol in the kit was followed using the PCR product from above. After the purification, Nano drop readings were then taken to determine the concentration of the DNA (ng/uL)(Table M1). Before beginning restriction digestion, the pTYB12 vector had to be isolated using a QIAprep Spin Miniprep kit (250) Cat. No 27106 according to protocol. Nano-drop readings were also taken to determine the concentration of pTYB12 vector that had been isolated (Table M2).

Restriction Digestion/Ligation

Restriction digestion was set up by adding 42uL of clean PCR product of complexin 1,2,3, and 4 to each of 4 tubes and adding 42uL of vector pTYB12 to three tubes. 5uL of 10x Ecor1 buffer, 1.5uL of Nde1 (20,000 units/mL) restriction enzyme, and 1.5uL of EcoR1 (20,000 units/mL) restriction enzyme were added to each of the 7 tubes. The seven tubes were incubated for 2 hours @ 37°C, after two hours, the pTYB12 vector was heat inactivated at 65°C for 20 min while the other 4 tubes remained in the incubator. After heat activation, the pTYB12 vector was treated with 1uL of CIP enzyme

and placed back in the incubator for 1 more hour. After the hour was complete, the products were mixed with 10uL of loading dye and run on a 1% agarose gel at 100V for 1 hour.

Next was gel extraction. The gel was placed under UV light and the digested products were cut from the gel and placed in 1.5 mL tubes. Protocol was then followed according to the QIAquick Gel Extraction Kit (50) Cat. No. 28704. After the elution of the DNA using 50uL of HPLC water, Nano drop readings were again taken to determine the concentration of the Digested DNA. (Table M2)

Ligation of the complexin isoforms and vector pTYB12 was set up using a 10uL reaction mixture in a PCR tube. One uL of T4 DNA ligase (#M0202S New England BioLabs) and 1uL of 10x buffer for T4 DNA ligase (#B0202S New England BioLabs) was then added to each tube. The amount of vector and insert that needed to be added was determined by using 2 formulas: $\text{Insert ng} = 4[\text{BP insert}/\text{BP vector}] \times \text{vector ng}$, and $\text{ng of insert} + \text{ng of vector} = 100\text{ng}$. HPLC water was added to the tubes in order to make the reaction mixture total 10uL. The ligation was set up according to Table M3. Once the mixtures were complete, the PCR tubes were placed in the PCR machine and incubated at 17°C overnight and then placed in the 4°C fridge until they were to be used for transformation into Novablue. (NOTE: from table M2, pTYB 12 was used for complexin 2 and 3 and pTYB 12 #1 was used for complexin 1 and pTYB 12 #2 was used for complexin 4)

Transformation into Novablue

After ligation of the complexin isoforms with the pTYB12 vector, the ligation product was transformed into competent *Escherichia coli* Novablue cells (Novagen). The

Novablue cells were removed from the -70°C freezer and thawed on ice for about 5 minutes. The cells were then placed in cold 1.5ml micro-centrifuge tubes in 15uL aliquots. 1.5uL of each ligation product was placed in its respective tube and mixed with cells by gently flicking. The mixture was then left to incubate in ice for 30 minutes, then heat shocked at 42°C in a heating block for 30 seconds, then put back in the ice for 10 more minutes. 75uL of SOC media was then added to each tube and then incubated at 37°C for 1 hour. After the 1-hour incubation, mixture was placed on LB/Ampicillin (100ug/mL). The cells were spread out on the plates by shaking the plates back and forth with glass beads on them. The glass beads were removed and the plates were placed in the incubator to incubate overnight at 37°C (incubated upside down for the first 20 minutes).

Plasmid Isolation/Sequencing

The following day, colonies were picked for sub-culturing in order to do plasmid isolation. Colonies from each transformation were placed in test tubes with 5mL of LB/ampicillin (100ug/ml) broth and grown overnight in a 37°C incubator. After the overnight growth, plasmid isolation of the transformed cells was done using a QIAprep Spin Miniprep kit (250) Cat. No 27106 according to protocol. After the isolation, Nano-drop reading were taken in order to determine the concentration of the plasmid and the plasmids were labeled and stored in the -20°C freezer.

Using the primer HXO_C63 designed specifically for our plasmids, we prepared and sent our plasmids off for sequencing. The sequencing mixture consisted of 7uL(or 500-1000ng) of DNA, 2uL of primer, and 3uL of HPLC water (to make total mixture 12uL). Once we got the results back, we ran a nucleotide BLAST on the NCBI website

of our sequence results with the predicted sequence of our complexin isoforms to determine if we had any errors in our sequences. After confirming the sequences of complexin 1, 2, 3, and 4, we then proceeded to transform the plasmids into *E. coli* Rosetta 2(DE3) competent cells. (Novagen)

Transformation into Rosetta 2 (DE3)

Rosetta 2 (DE3) cells were taken out of the -70°C freezer and thawed on ice for about 5 minutes. These cells were then placed into a 1.5mL micro-centrifuge tube in 10uL aliquots. 1ul of each plasmid was then added to its respective tube that had 10uL of Rosetta 2 cells and incubated on ice for 30 min. The cells were then heat shocked in a heating block at 42°C for 30 seconds and placed back in the ice to incubate for 10 more minutes. 90uL of LB broth was added to each tube and incubated at 37°C for 1 hour. 50uL of the cells were then put on an LB/ampicillin (100ug/mL)/chloramphenicol (34ug/mL) plate and rolled with glass beads to spread out the bacteria. The plates were then incubated overnight at 37°C.

Small-Scale Induction

Small-scale induction of the complexin 1, 2, 3, and 4 isoforms was done by making overnight cultures from the transformation of the vector and isoform into Rosetta 2 (DE3). First, OD₆₀₀ readings were taken from the overnight cultures. Using these readings and the formula $C_1V_1=C_2V_2$ each culture was normalized to .1 in 10mL of LB both with 10uL of Ampicillin 100 and 10uL of Chloramphenicol 34. Each culture was then split into 2 duplicates of 5mL each. One duplicate was labeled with a “+” and the other duplicate with a “-” to indicate which will be induced with IPTG. These duplicates were placed in the incubator at 37°C/220rpm for 2 hours. After 2 hours, the

OD₆₀₀ was taken again and if these readings were between .4 and .8 then 2.5uL of IPTG was added to the tubes labeled with a +. After adding IPTG the tubes were placed back into the incubator at 37°C/220rpm for 4 more hours. After the 4-hour incubation, the OD₆₀₀ was taken again. Samples were then taken for analysis on SDS-PAGE

SDS-PAGE

SDS-PAGE gels were created as the following:

Table 2 - 10% SDS-PAGE gels

10% SDS-PAGE Gels (Bottom Layer)	
Millipore Water	7.9mL
1.5M Tris [pH 8.8]	5.0mL
30% acrylamide	6.7mL
10% SDS	200μL
10% Ammonium Persulfate	200μL
TEMED	8μL

Table 3 - 5% stacking layer

5% Stacking Layer	
Millipore Water	5.5mL
1.0M Tris [pH 6.8]	1.0mL
30% acrylamide	1.3mL
10% SDS	80μL
10% Ammonium Persulfate	80μL
TEMED	8μL

Bottom Layer mixed together, after addition of TEMED, 4.5mL of solution was poured into 1mm glass plates and covered with a layer of isopropanol until solidified. Isopropanol was removed by rinsed with distilled water, and 5% stacking layer was poured on top with appropriate combs for wells inserted. Used once solidified.

Samples were prepared for SDS-PAGE through the following: 10D of cell culture spun down in 1.5mL microcentrifuge tube, then resuspended in 2x SDS sample Buffer by vortexing. For lysis, 0.5mm glass beads were added and vortexed for 1min

after addition of 1mM PMSF. Samples were boiled @ 95°C for 5min. Samples cooled to room temperature before loading.

Samples loaded in gel in appropriate manner. 10µL of Broad Range Standard (BioRad), and 15µL of samples.

Run at 150V/1hr in BioRad Mini-PROTEAN Tetra System.

For staining, the gels were soaked in Fixing Solution for 30min (45% methanol, 10% acetic acid), the Coomassie Blue Staining Solution (45%methanol, 10%acetic acid, 0.05% w/v Coomassie Brilliant Blue R-250) for 1 hr, then destained overnight in Destaining Solution (5% methanol, 7% acetic acid)

Chapter 3: Data and Results

We used the complexin 1 and 2 cDNA obtained from rat and the complexin 3 and 4 cDNA obtained from mouse and amplified it using PCR. We then took the amplified DNA specific for each complexin isoform and did restriction digestion and ligation to the pTYB12 vector so the plasmid could be transformed into *E.coli* for expression. After expression the plasmids were re-isolated for sequencing and transformation into Rosetta 2 (DE3) competent cells for induction.

PCR results

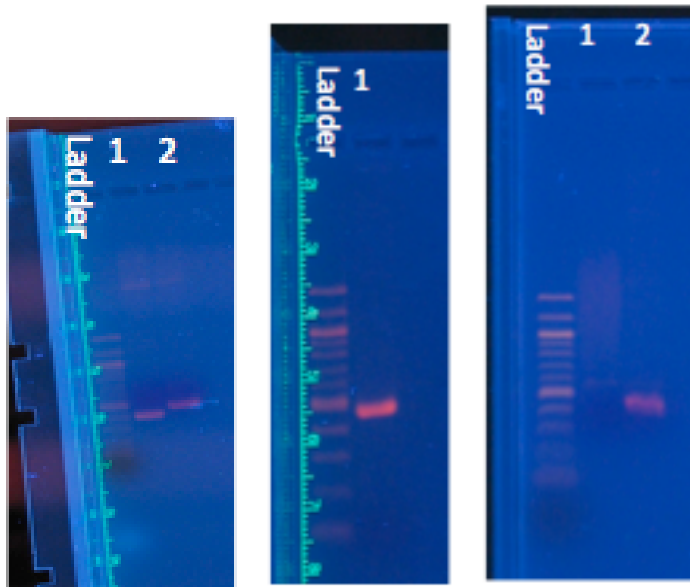


Figure 5: Left: Visible band of the complexin 1 PCR product in well 1 and complexin 4 product in well 2. **Middle:** Visible band of complexin 2 PCR product in well 1. **Right:** Visible band of complexin 3 PCR product in well 2.

Table M1 - Nano-drop readings after PCR cleanup

	ng/uL	260/280	260/230
Complexin I	37.1	1.94	1.96
Complexin II	17.8	1.93	2.09
Complexin III	36.6	1.89	2.12
Complexin IV	43.3	1.87	2.09

Table M2 - Nano-drop reading after gel extraction/pTYB12 vector isolation

	ng/uL	260/280	260/230
Complexin I	12.6	2.14	0.07
Complexin II	8.9	2.09	0.03
Complexin III	18.9	2.05	0.05
Complexin IV	19.3	1.84	0.15
pTYB 12	21.5	1.90	0.03
pTYB 12 #1	15.3	2.18	0.07
pTYB 12 #2	13.7	1.94	0.10

Table M3 - ligation set up

	Complexin I	Complexin II	Complexin III	Complexin IV
T4 Ligase	1uL	1uL	1uL	1uL
10x buffer	1uL	1uL	1uL	1uL
pTYB 12 vector	5.3uL	3.7uL	3.7uL	5.9uL
Insert	1.4uL	2.2uL	1.1uL	1.1uL
HPLC water	1.2uL	2.1uL	3.2uL	1uL

Sequencing results

gi|402692190:75-479 Rattus norvegicus complexin 1 (Cplx1), mRNA

Sequence ID: lcl|24595 Length: 405 Number of Matches: 1

Range 1: 1 to 405 [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
743 bits(402)	0.0	404/405(99%)	0/405(0%)	Plus/Plus
Query 112	ATGGAGTTCGTGATGAAACAAGCCCTGGGAGGGGCCACCAAGGATATGGGGAAGATGCTT			171
Sbjct 1	ATGGAGTTCGTGATGAAACAAGCCCTGGGAGGGGCCACCAAGGATATGGGGAAGATGCTT			60
Query 172	GGGGGTGATGAGGAGAAGGACCCCGATGCCGCCAAGAAGGAGGAGGAGCGGCAGGAGGCCA			231
Sbjct 61	GGGGGTGATGAGGAGAAGGACCCCGATGCCGCCAAGAAGGAGGAGGAGCGGCAGGAGGCCA			120
Query 232	CTGCGGCAGGCAGAGGAGGAGCGCAAAGCAAAGTATGCCAAGATGGAGGCAGAACGTGAG			291
Sbjct 121	CTGCGGCAGGCAGAGGAGGAGCGCAAAGCAAAGTATGCCAAGATGGAGGCAGAACGTGAG			180
Query 292	GTCATGCGGCAGGGCATAACGAGATAAGTATGGCATCAAGAAGAAGGAGGAGCGTGAGGCT			351
Sbjct 181	GTCATGCGGCAGGGCATAACGAGATAAGTATGGCATCAAGAAGAAGGAGGAGCGTGAGGCT			240
Query 352	GAGGCTCAGGCAGCCATGGAGGCCAACTCAGAAGGCAGCCTGACTCGACCCAAGAAGGCT			411
Sbjct 241	GAGGCTCAGGCAGCCATGGAGGCCAACTCAGAAGGCAGCCTGACTCGACCCAAGAAGGCT			300
Query 412	ATCCCACCAGGCTGTGGGGACGAGCCCGAGGAGGAAGATGAGAGCATCCTGGACACTGTC			471
Sbjct 301	ATCCCACCAGGCTGTGGGGACGAGCCCGAGGAGGAAGACGAGAGCATCCTGGACACTGTC			360
Query 472	ATCAAGTACCTGCCTGGGCCACTGCAGGACATGTTCAAGAAGTAA		516	
Sbjct 361	ATCAAGTACCTGCCTGGGCCACTGCAGGACATGTTCAAGAAGTAA		405	

Figure 6: Complexin 1 sequencing Results matched with Rat complexin 1 using a Nucleotide BLAST on the NCBI website

gi|12408324|ref|NP_074055.1| complexin-1 [Rattus norvegicus]

Sequence ID: lcl|6247 Length: 134 Number of Matches: 1

Range 1: 1 to 134		Graphics	▼ Next Match ▲ Previous Match		
Score	Expect	Method	Identities	Positives	Gaps
261 bits(668)	3e-95	Compositional matrix adjust.	134/134(100%)	134/134(100%)	0/134(0%)
Query	1	MEFVMKQALGGATKDMGKMLGGDEEKDPDAAKKEEERQEALRQAEERKAKYAKMEAEERE	60		
Sbjct	1	MEFVMKQALGGATKDMGKMLGGDEEKDPDAAKKEEERQEALRQAEERKAKYAKMEAEERE	60		
Query	61	VMRQGIRDKYGIKKKEEREAEQAAMEANSEGLTRPKKAIPPGCGDEPEEEDSILDTV	120		
Sbjct	61	VMRQGIRDKYGIKKKEEREAEQAAMEANSEGLTRPKKAIPPGCGDEPEEEDSILDTV	120		
Query	121	IKYLPGLQDMFKK	134		
Sbjct	121	IKYLPGLQDMFKK	134		

Figure 7: Protein BLAST of complexin 1 to confirm amino acid sequence

The analysis of the sequencing results by running a protein BLAST of the amino acid sequence of the complexin 1 template and the subject showed that the mutation from a C to a T was not significant and the amino acid sequence remained conserved, confirming our product.

gi|16758743:295-699 Rattus norvegicus complexin 2 (Cplx2), mRNA

Sequence ID: lcl|215557 Length: 405 Number of Matches: 1

Range 1: 1 to 405		Graphics	▼ Next Match ▲ Previous Match		
Score	Expect	Identities	Gaps	Strand	
739 bits(400)	0.0	403/405(99%)	0/405(0%)	Plus/Plus	
Query	114	ATGGACTTCGTCATGAAGCAGGCCCTCGGAGGGGCCACCAAGGACATGGGGAGAGATGCTG	173		
Sbjct	1	ATGGACTTCGTCATGAAGCAGGCCCTCGGAGGGGCCACCAAGGACATGGGGAGAGATGCTG	60		
Query	174	GGGGagaggaggagaaggagacccagatgcacagaaagaaggagggagcggcaggagggcc	233		
Sbjct	61	GGGGAGAGGAGGAGAGAAGGACCCAGATGCACAGAAGAAGGAGGAGGAGCGGCAGGAGGCC	120		
Query	234	ctgaggCAGCAGGAGGAAGAGCGCAAGGCCAAACATGCCCGCATGGAAGCGGAACGTGAG	293		
Sbjct	121	CTGAGGCAGCAGGAGGAAGAGCGCAAGGCCAAACATGCCCGCATGGAAGCGGAACGTGAG	180		
Query	294	AAGGTCCGGCAGCAGATTTCGAGATAAGTATGGGCTGAAGAAGAAAAGAGAAGGAGGCCA	353		
Sbjct	181	AAGGTCCGGCAGCAGATTTCGAGATAAGTATGGGCTGAAGAAGAAAAGAGAAGGAGGCCA	240		
Query	354	GAGGAGAAGGCAGCCCTGGAACAGCCCTGCGAGGGGAAGCCTGACCAGACCCAAGAAGGCC	413		
Sbjct	241	GAGGAGAAGGCAGCCCTGGAACAGCCCTGCGAGGGGAAGCCTGACCAGACCCAAGAAGGCC	300		
Query	414	ATCCCTGCAGGCTGTGGGGACGAGGAGGAGGAAGAAGAGGAGAGCATCCTGGACACGGTG	473		
Sbjct	301	ATCCCTGCAGGCTGTGGGGACGAGGAGGAGGAAGAAGAGGAGAGCATCCTGGACACGGTG	360		
Query	474	CTCAAATATCTGCCGGGGCCACTGCAGGACATGTTCAANAAGTAA	518		
Sbjct	361	CTCAAATATCTGCCGGGGCCACTGCAGGACATGTTCAAGAAGTAA	405		

Figure 8: Complexin 2 sequencing Results matched with Rat complexin 2 using a Nucleotide BLAST on the NCBI website

gi|16758744|ref|NP_446330.1| complexin-2 [Rattus norvegicus]

Sequence ID: lcl|147751 Length: 134 Number of Matches: 1

Range 1: 1 to 134 [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
257 bits(656)	2e-93	Compositional matrix adjust.	133/134(99%)	133/134(99%)	0/134(0%)
Query 1	MDFVVKQALGGATKDMGKMLGEEEEKDPDAQKKEEERQEALRQEEERKAKHARMEAERE				60
Sbjct 1	MDFVVKQALGGATKDMGKMLGEEEEKDPDAQKKEEERQEALRQEEERKAKHARMEAERE				60
Query 61	KVRQQIRDKYGLKKKEEKEAEEKAALQPCESLTRPKKAIPAGCGDEEEEEESILDTV				120
Sbjct 61	KVRQQIRDKYGLKKKEEKEAEEKAALQPCESLTRPKKAIPAGCGDEEEEEESILDTV				120
Query 121	LKYLPGPLQDMFKK 134				
Sbjct 121	LKYLPGPLQDMF K 134				

Figure 9: Protein BLAST of complexin 2 to confirm the amino acid sequence

The analysis of the sequencing results by running a protein BLAST of the amino acid sequence of the complexin 2 template and the subject showed that the one mutation from an A to a G was not significant and the amino acid sequence remained conserved, confirming our product. Also, the N at base 512 in the sequencing results for complexin 2 was determined to be a G meaning that there was no mutation there. This N corresponds with amino acid 133, which is why the protein BLAST of complexin 2 has an X at amino acid 133. Since there was no nucleotide mutation there was also no amino acid mutation.

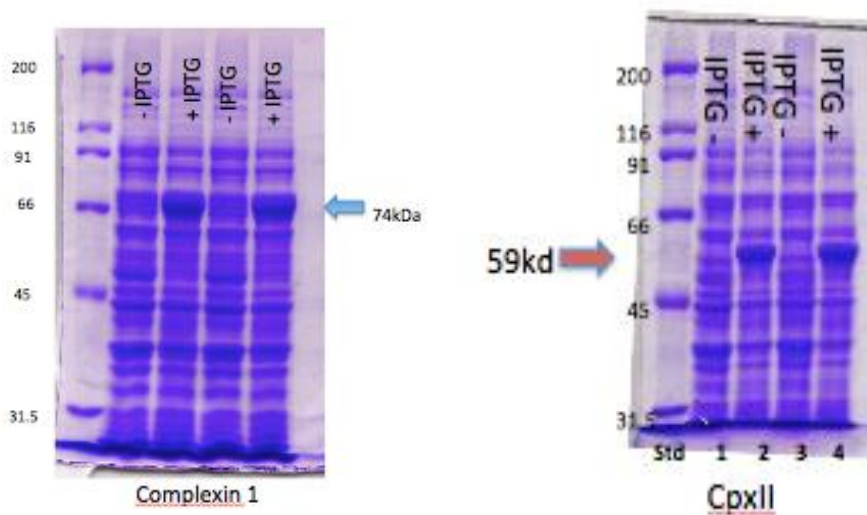
gi|256574830:138-614 *Mus musculus* complexin 3 (Cplx3), mRNA
 Sequence ID: lcl|43255 Length: 477 Number of Matches: 1

Range 1: 1 to 477		Graphics		Next Match	Previous Match
Score	Expect	Identities	Gaps	Strand	
881 bits(477)	0.0	477/477(100%)	0/477(0%)	Plus/Plus	
Query	110	ATGGCGTTCATGGTGAAGTCCATGGTGGGTGGCCAACTGAAGAACCTCACAGGGAGCCTG			169
Sbjct	1	ATGGCGTTCATGGTGAAGTCCATGGTGGGTGGCCAACTGAAGAACCTCACAGGGAGCCTG			60
Query	170	GGGGGCGGCGAGGACAAGGGGGACGGAGACAAGTCTGCAGCCGAAGCACAGGGCATGAGC			229
Sbjct	61	GGGGGCGGCGAGGACAAGGGGGACGGAGACAAGTCTGCAGCCGAAGCACAGGGCATGAGC			120
Query	230	CGAGAAGAGTACGAGGAGTATCAGAAGCAACTGGTGGAGGAGAAGATGGAGCGAGATGCA			289
Sbjct	121	CGAGAAGAGTACGAGGAGTATCAGAAGCAACTGGTGGAGGAGAAGATGGAGCGAGATGCA			180
Query	290	CAGTTCACACAGAGGAAGGCAGAGCGGGCCACACTGCGAAGTCACTTCAGAGACAAATAC			349
Sbjct	181	CAGTTCACACAGAGGAAGGCAGAGCGGGCCACACTGCGAAGTCACTTCAGAGACAAATAC			240
Query	350	CGTCTGCCCAAGAACGAAACAGATGAAAGCCAGATCCAGCTGGCAGGCGGAGATGTGGAG			409
Sbjct	241	CGTCTGCCCAAGAACGAAACAGATGAAAGCCAGATCCAGCTGGCAGGCGGAGATGTGGAG			300
Query	410	CTGCCTCGGGAGCTAGCCAAGATGATCGAGGAGGATACGGAGGAAGAGGAGGATAAGGCC			469
Sbjct	301	CTGCCTCGGGAGCTAGCCAAGATGATCGAGGAGGATACGGAGGAAGAGGAGGATAAGGCC			360
Query	470	TCTGTGCTCGGGCAGCTGGCCAGCCTCCCTGGCCTAGACCTCAGCTCACTCAAGGACAAA			529
Sbjct	361	TCTGTGCTCGGGCAGCTGGCCAGCCTCCCTGGCCTAGACCTCAGCTCACTCAAGGACAAA			420
Query	530	GCCCAGACTACACTAGGGGACCTCAAGCAATCAGCTGAGAAGTGCCATATCATGTGA			586
Sbjct	421	GCCCAGACTACACTAGGGGACCTCAAGCAATCAGCTGAGAAGTGCCATATCATGTGA			477

Figure 10: Complexin 3 sequencing Results matched with mouse complexin 3 by using a Nucleotide BLAST on the NCBI website

Analysis of the sequencing results confirmed that the complexin 3 was successfully transformed into *E.coli* without any errors in the DNA.

SDS-PAGE results



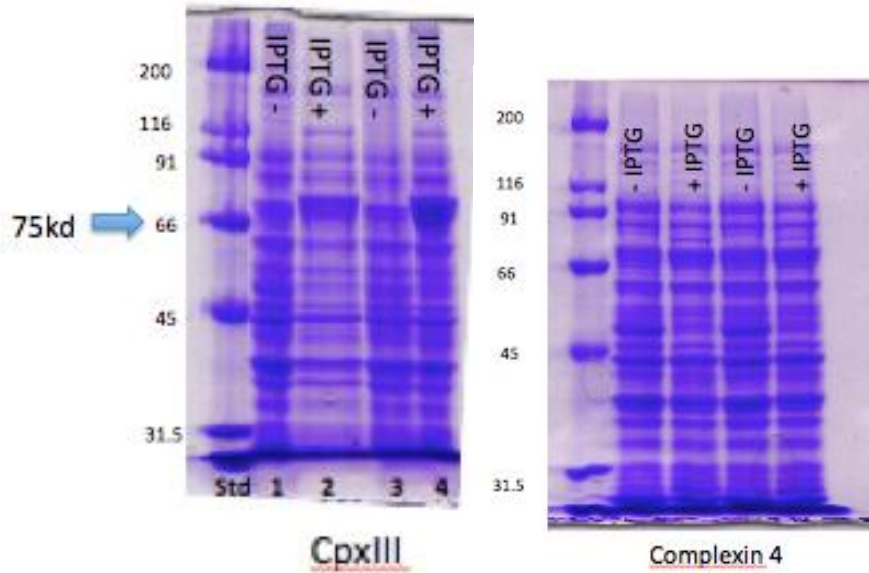


Figure 11: SDS-PAGE results for each complexin isoform. Complexin 1 had a visible band at 75kDa confirming expression. Complexin 2 had a visible band at 59kd. This was the wrong size for the vector and insert. Complexin 3 had a visible band at 75kd confirming expression. There was no visible band for complexin 4.



Figure 12: PCR confirmation of complexin 4 plasmid after no results were returned from sequencing.

Chapter 4: Discussion

In order to be able to use an *in vitro* fusion assay to study the roles of the four complexin isoforms in regulation of SNARE-mediated fusion, each isoform had to be purified. This was done through cloning into an expression vector, then transformation into *E. coli* Rosetta 2 (DE3) expression strain. Using the T7 expression system, and IPTG induction allowed us to verify expression of these isoforms in *E. coli*. Each of these isoforms was tagged with a chitin-binding domain that is present in the pTYB12 vector, which will allow for specific purification of each isoform by using the NEB IMPACT system.

We were successful in amplifying the DNA for all the complexin isoforms by doing a PCR as seen in figure 5. After PCR, we digested the PCR product and the pTYB12 vector with restriction enzymes NdeI and EcoRI to prepare for ligation. After ligation, the recombinant plasmid with the vector and complexin isoform was transformed into *E. coli* for expression. We then re-isolated the plasmid from the *E. coli* to send it off for sequencing to ensure we had the correct insert without any errors.

We were able to confirm the sequences for complexin 1, 2, and 3. In order to confirm the sequences for complexin 1 and 2, we had to run a protein BLAST of the template protein sequence with the translated sequence of both complexin 1 and 2 to ensure that there was not an error in the amino acid sequence even though there were errors in the DNA sequence as seen in figures 6-10. After multiple attempts we have still not received a complete, confirmed sequence from the sequencing company of complexin 4. The first time we sequenced it, we did not get any results back. So we ran a PCR of the recombinant plasmid to be sure that the insert was there. The PCR confirmed that the

insert was indeed there (figure 12), and there must have been an issue with our sequencing mixture so we isolated the plasmid again and sent it off for sequencing. Of the results we have obtained, the sequences are still 60-120 base pairs short for complexin 4.

After confirmation of the other isoforms, we ran a small-scale induction and used IPTG to induce T7 promoter and the production of the complexin isoforms. After induction, the samples were run on an SDS-PAGE gel to confirm expression of each isoform. Even though, we did not get the entire sequence for complexin 4, we went ahead and did a small-scale induction and SDS-PAGE gel run due to the small chance that there was an error in the sequence because the gene is so small and the fact that the PCR confirmed that the insert for the protein was present. Of the four isoforms that were run, only Complexin 1 and 3 were confirmed at 75kD. Complexin 2 was the wrong size at 59kD and there was no visible band for the Complexin 4 sample. The band at 59kD for complexin 2 is the size of the vector without the insert. Even though we confirmed the sequence for complexin 2 after plasmid isolation, the band on the SDS-PAGE gel was not the right size.

In conclusion, after cloning and expression of all of the Complexin isoforms, we were only able to successfully confirm and induce Complexin 1 and 3. We are currently working on going back and cloning Complexin 2 and 4 for expression and induction. Once we successfully clone and induce the production of all of the isoforms, each one will be isolated using the NEB IMPACT protein purification system to obtain the Complexin 1, 2, 3, and 4 isoforms in order to study their regulatory function in SNARE-mediated mast cell degranulation.

Literature Cited

1. McNew, James A. "Regulation of SNARE-Mediated Membrane Fusion during Exocytosis" *Chem Rev.* 2008, 108, 1669-1686
2. Weninger, Keith R. "Complexin arrests a neighbor" *Natural America, Inc.* 2011; 861-863
3. Gilfillan, Alasdair M. & Beaven, Michael A. "Regulation of mast cell responses in health and disease" *Crit Rev Immunol.* 2011; 31(6), 475-529
4. Blank, Ulrich "The Mechanism of Exocytosis in Mast Cells" *Mast Cell Biology* 2011; 107-122
5. Harvima, Ilkka., & Nilsson, Gunnar. "Mast Cells as Regulators of Skin Inflammation and Immunity" *Acta Derm Venerol* 2011
6. Kindt, T. J., Goldsby, R. A., Osborne, B. A., Kuby, J., & et al, J. (2007). *Kuby immunology.* (6th ed.). New York: W. H. Freeman.
7. Brose, Nils "For Better or for Worse: Complexin regulates SNARE Function and Vesicle Fusion" *Traffic* 2008 9: 1403-1413
8. Tadokoro, S., Nakanishi M., and Hirashima, N. "Complexin II facilitates exocytotic release in mast cells by enhancing Ca²⁺ sensitivity of the fusion process" *Journal of Cell Science* 2005. 118, 2239-2246
9. *IMPACT- CN System.* (n.d.). Retrieved from http://www.ebiotrade.com/buyf/productsf/Cell%20Signaling%20Technology/relation/IMPACT_CN_Sys.pdf