CHARACTERIZATION OF SECRETOGRANIN III IN MAST CELLS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MICROBIOLOGY

AUGUST 2006

By

Prerna Prasad

Thesis Committee:

Helen Turner, Chairperson

Claude Jourdan-Le Saux

Sandra Chang

Alexander James Stokes

We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Microbiology.

THESIS COMMITTEE

Chairperson

Acknowledgements

First and foremost, I would like to thank and dedicate my thesis to Dr. Helen Turner, my advisor, for her constant and persistent guidance with my research, in spite of her busy schedule. I truly value her advice and interest in my project.

Next I would like to thank my wonderful mother without whose sacrifices and support, I would have never been able to come this far.

Special thanks go to Dr. Alexander James Stokes, who consistently helped my research and also agreed to be a part of my Master's defense committee. He generously developed the SgIII-V5 construct for my research.

I am also grateful to all my lab members – Kimberly del Carmen, Clay Wakano, Lori Shimoda, Li Zengqiu, Andrea Small Howard, Cora Rillero, Linden T. Doescher, Kimberly E. M. Cochrane, Janos Molnar, Michael Canute and Martin Liss, for their help and support. I have sincerely enjoyed working with all of them over the last two years at Laboratory of Cell Biology and Immunology.

I would like to acknowledge and thank my committee members - Dr. Claude Jourdan-Le Saux and Dr. Sandra Chang for their valuable comments and suggestions.

Last but not the least, I would like to thank Arasu for his encouragement, love, and incredible support. He truly made me believe I could accomplish this goal and I look forward to spending the rest of my life with him.

Abstract

Mast cells are granular immunocytes that reside in the body's barrier tissues. These cells control the initiation, and maintenance, of protective inflammatory responses to immune challenges. Mast cells secrete inflammatory mediators including histamine and matrix-active proteases from specialized, membrane-delimited, intracellular vesicles. The abundance and load of these vesicles are direct determinants of the intensity of inflammatory responses, and are therefore potential control points for inflammatory diseases such as asthma and eczema. However, the proteins that control granule behavior in mast cells are not yet fully understood. Dr. Turner has previously used transcriptional arrays to identify genes whose products are likely to be involved in mast cell secretion. Secretogranin III (SgIII) was identified for our study, as this protein showed an intriguing expression profile after FcεRI stimulation of mast cells. SgIII transcript levels were transiently up-regulated following induction of secretion, like the mast cell secreted proteases, which are known components of the secretory vesicles. Moreover, SgIII was the only protein among its family members that showed this expression pattern. We have shown that SgIII is expressed in mast cells at the protein level and is organized into vesicular structures. Our results show that over-expression of SgIII in mast cells is sufficient to cause an expansion of a vesicular compartment in these cells. In mast cells, these novel granules exhibit some features reminiscent of secretory vesicles, such as the storage of inflammatory mediators that can then be released in response to physiological stimuli, indicating that they may also function as *bona fide* secretory vesicles.

Table of Contents

List of Tables

List of Figures

List of Abbreviations.

ATP: Adenosine 5'-triphosphate

CCD: Charge-coupled device

cDNA: complementary DNA

CgA: Chromogranin A

CgB: Chromogranin B

CgC: Chromogranin C

CMV: cytomegalovirus

CPE: Carboxypeptidase E

cpm: counts per minute

C-terminus: carboxyl-terminus

DMEM: Dulbecco's modification of Eagle's medium

DNA: deoxyribonucleic acid

DNP-BSA: Dinitrophenyl-Bovine serum albumin

ECL: enhanced chemiluminescence

EDTA: ethylenediamine tetra-acetic acid

FBS: Fetal bovine serum

Fc: Fragment crystallizable

FcεRI: Fcepsilon receptor I

GAPDH: Glyceraldehyde –3- phosphate dehydrogenase

HEK293: Human embryonic kidney cell line 293

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

h: hour

HRP: Horseradish peroxidase

Ig: immunoglobulin

IL: Interleukin

kDa: Kilo Dalton

min: minute

MLMC: Mucosal like mast cells

mRNA: messenger RNA

NGF: Nerve growth factor

N-terminus: Amino-terminus

PAF: platelet activating factor

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PC12: rat pheochromocytoma cell line 12

PCR: polymerase chain reaction

PMA: Phorbol myristate acetate

PMSF: phenylmethylsulfonyl fluoride

PVDF: polyvinylidene difluoride

RBL: rat basophilic leukaemia

RBL2H3M1: RBL2H3 cells with stable over-expression of M1 mucosamine acetyl choline receptor

RNA: ribonucleic acid

RNAse: Ribonuclease

RNAi: RNA interference

RT: room temperature

SDS: sodium dodecyl sulfate

SgI: Secretogranin I

SgII: Secretogranin II

SgIII: Secretogranin III

SgIV: Secretogranin IV

SgV: Secretogranin V

SgVI: Secretogranin VI

siRNA: small interfering RNA

TBS: Tris buffered saline

TGN: Trans Golgi Network

TTBS: Tween 20 + tris buffered saline

TNFα: Tumor necrosis factor alpha

/TO: tetracycline off

Tris: tris (hydroxymethyl) aminomethane

WB: Western Blot

Chapter 1. Introduction

Our immune system is primed to protect us against infective and opportunistic pathogens. One of our primary defense mechanisms against pathogens is the induction of an inflammatory response. In our barrier tissues, mast cells serve as reservoirs of several potent biological mediators that are normally only released to combat infective parasites or micro-organisms (1-5). When inappropriately activated, these mast cells are responsible for causing pathological inflammatory disorders, such as asthma, allergy, eczema and urticaria (2, 3, 6).

1.1. Mast cells are Granular Leukocytes.

1.1.1. Sentinels of the immune system

Paul Ehrlich was the first to describe "Mastzellen", meaning "well fed" cells, in his doctoral thesis "Contributions to the Theory and Practice of Histological Staining" (1878) as having unique histological staining characteristics and a granular cytosol. These cells, now known as mast cells, have fascinated the immunological community.

Mast cells are derived from a multipotent $CD34^+$ precursor in the bone marrow and circulate in the peripheral blood as agranular monocytic cells (3, 4, 7, 8). After migrating into tissues, these immature mast cells become terminally differentiated, assume their typical granular morphology and express high surface levels of their antigen receptor, FcεRI. Under normal conditions, mature mast cells are not seen in the peripheral circulation.

A protective function for mast cells in parasitic diseases has been proposed on the basis of some worm infection models (16). Recent findings in animal models of bacterial infection suggest that mast cells may have a protective role in host defense against pathogens in innate immunity along with the probable role of mast cells in acquired

immunity against parasitic infection (2, 8, 16, 20). Mast cells are frequent participants in non-allergic immune responses as well as in allergic or IgE mediated responses. They have a diversity of surface receptors and effector capabilities that detect, and deliver a first response to invading bacteria, and other insults. They may be actively accumulated within inflamed tissues, producing cytokines and other mediators that contribute vitally to ongoing inflammations.

1.1.2. Mast cells are mediators of inflammation and allergy.

Mast cells express a high affinity receptor for the Fc portion of Immunoglobulin E (IgE) called FcεRI. When the body is exposed to allergens such as plant pollens, drugs, allergen specific IgE binds to these receptors, causing these cells to become sensitized. Upon challenge, membrane bound IgE/ FcεRI complexes are cross-linked by allergens, thereby triggering degranulation of mast cells and the release of inflammatory mediators such as histamine (11-14). These mediators also cause the wheezing of asthma, the itching of eczema, the sneezing of hay fever and, in extreme cases, the life-threatening shock of anaphylaxis. Although this reaction is most well understood in terms of allergy, it appears to have evolved as a defense system against intestinal worm infestations.

Inflammation is classically characterized by *rubor* (reddening due to vasodilation), *tumor* (swelling due to oedema), *calor* (heat due to intense metabolic activity of infiltrating leukocytes and increased blood flow) and *dolor* (pain caused by the effects of inflammatory mediators on local sensory nerve endings). Mast cell activation can be induced by a variety of stimuli, including (1) innate/adaptive immune challenges, (2) neuronally derived mediators such as NGF, (3) secretagogues such as polybasic mastoparan peptide found in the wasp venom, and (4) physical stimuli such as heat and mechanical pressure (3, 4, 9, 10). Mast cells contain cytoplasmic granules that store and release inflammatory mediators such as histamine, some cytokines, proteases and others

(summarized in Figure 1). These granules are structurally, and functionally, related to the dense core vesicles of neuro-endocrine cells and neurons.

Extra-cellular release of pro-inflammatory mediators such as histamine causes relaxation of vascular smooth muscle, leading to vasodilation. The action of histamine, platelet activating factor (PAF) and lipid mediators upon the vascular endothelium leads to increased vascular permeability, thereby causing fluid extravasation, and hence an increase in the hemostatic pressure of the affected tissue. Mast cell-derived chemokines, and cytokines, drive the recruitment of the leukocyte infiltrate that is primarily neutrophilic with some lymphocyte components. The release of serotonin and histamine, from mast cells directly sensitizes sensory neurons, leading to the phenomenon of inflammatory hyperalgesia, or pain. Finally, tissue remodeling is a consequence of growth factor release by mast cells that causes proliferation of fibroblasts and promotes neo-vascularization. In addition, mast cell derived proteases (e.g. chymase, tryptase, cathepsins) play a pivotal role in replacing a temporary, homogeneous, tissue substrate with a permanent, heterogeneous connective tissue matrix.

Mast cells, after degranulating and releasing mediators, are known to regranulate over a period of 1-6 h. This is said to be a recovery period for the granules and the granule contents (23). During this time the depleted granule contents are restored by induction of transcription and translation of the relevant genes, and cell morphology is restored. During this replenishment period mast cells are refractory to stimulation, but once fully recovered, they are ready to degranulate again. The capacity of mast cells to undergo several cycles of degranulation, and regranulation, is an important feature of these cells in the induction and perpetuation of an allergic reaction.

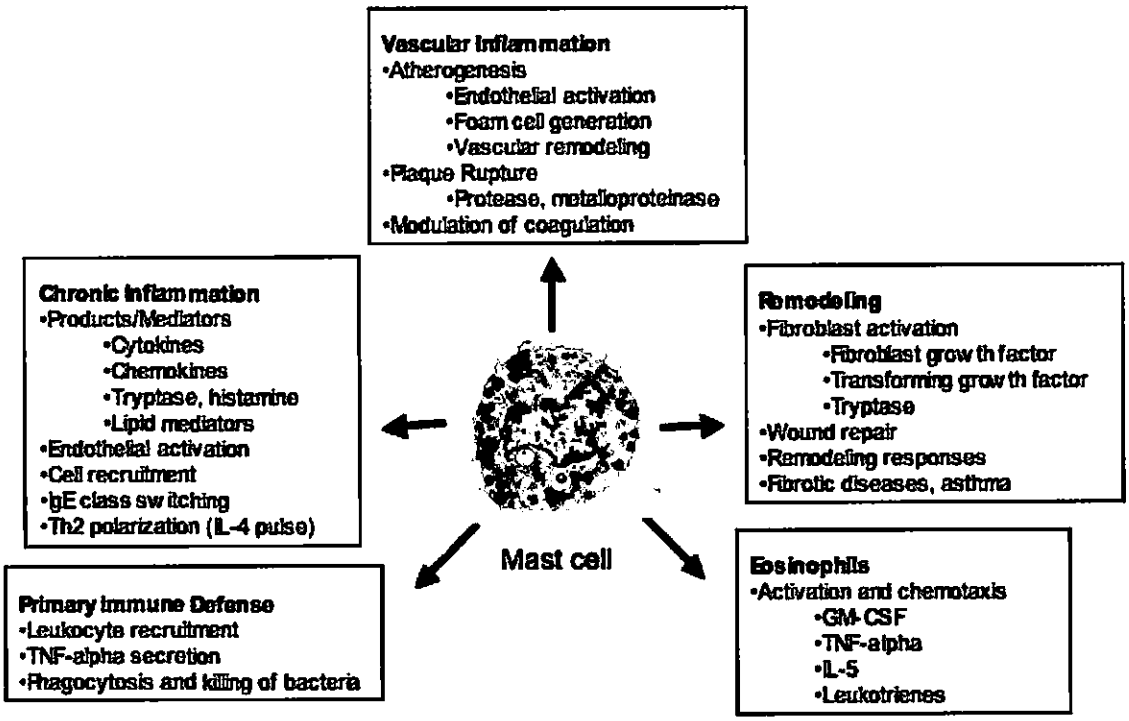


Figure 1. Biological functions of human mast cells.
Mediator and cytokine synthesis and regulation of human physiological and pathological processes.

1.1.3. Mast cells in wound healing

A wound is a disruption of tissue integrity. Wound healing relies on the induction of an inflammation where a tissue site is made hostile to pathogens by altered physical conditions and recruitment of phagocytes. The damaged tissue is repaired (necessitating re-vascularization and re-epithelialization), which is directed by mast cells. Mast cells have been shown to play a key role in dermal wound repair, and the presence of an infection delays wound healing (5, 21, 22).

Mast cells reside in the normal dermis and, the cytokines or growth factors they synthesize stimulate the fibroblasts to produce collagen which is important in fibrosis (1, 21, 22). They also contain serine proteases that are involved in remodeling of the extra-cellular matrix during healing. Mast cells are found in increased numbers in acute wounds and in certain chronic fibrotic diseases (21,22). Immediately after a wound has

been established, vasoactive substances, including histamine, serotonin, and cytokines are released which results in an increase in the vessel permeability resulting in an enhanced blood plasma leakage into tissue, leading to oedema. With leakage of blood plasma, other immunocytes, recruited by mast cell-derived chemokines, reach the wound and start the defense against infections and wound cleansing (1, 21, 22). The inflammatory exudate dilutes any toxins and prevents the spread of the inflammatory mediators to the surrounding tissues. Fibrinogen forms fibrin clots providing a mechanical barrier to the spread of microorganisms (if present) and additionally assists phagocytosis. It has been found that small, irregular mast cells (MLMC) accumulate in the wound, which suggest that these small MLMC migrate into the skin during wound healing, additionally to the resident population of mature mast cells (21). Frequently, there is some tissue destruction with inflammation and attempted healing occurring simultaneously.

1.1.4. Summary

- Mast cells mediate beneficial inflammations in host defense and wound healing.
- Mast cells mediate pathological inflammations in certain diseases such as rheumatoid arthritis, multiple sclerosis, asthma, fibrotic disorders and allergies.
- Our goal is to understand the molecular machinery that controls release of inflammatory mediators from mast cells.

1.2. The Granin Family components of the secretory machinery.

1.2.1. The Chromogranin-Secretogranin Family

The Granin family comprises a group of acidic proteins that are present in the secretory granules of a wide variety of endocrine and neuro-endocrine cells (24-27). The Chromogranin A (CgA), Chromogranin B (CgB) and Secretogranin II (SgII) are the members whose physiochemical properties have been best studied and identified. These proteins consist of 185 to 657 amino acid residues that include an amino-terminal signal peptide, which directs the protein towards the Golgi complex and the endoplasmic reticular lumen. These proteins, CgA, CgB, SgII, and the less well studied Secretogranin III-VII, are collectively referred to as 'Granins'. The Granins are also known to contain numerous pairs of basic amino acids with potential cleavage sites (24). The exact function of these proteins is not yet known. They may be the precursors of biologically active peptides and/or they may act as helper proteins in the packaging of peptide hormones and neuro-peptides. Apart from their sub-cellular location and the abundance of acidic residues (Asp and Glu), these proteins do not share many structural similarities, except a C-terminal motif, which is the only region that is conserved (27). Table 1 summarizes the physico-chemical properties of the Granin family members.

Property	CgA	CgB	SgII	SgIII	SgIV	SgV	SgVI
Chromosome localization	14(human), 21(bovine), 6(rat) 12(mouse)	20(human) 3(rat) 2(mouse)	2(human) 9(rat) 1(mouse)	2(mouse)	ND	15(human) 2(mouse)	20(human)
Amino acid residues	431-445	626-657	559-586	449-507	ND	185	241
Molecular mass (kDa)							
Calculated	49-52	48-52	67.5	51-57	ND	21	27.5
Apparent	74-80	100-120	86	57	35	23	55
Acidic residues (%)	25	24	20	19	ND	16	21
Isoelectric point (pHi)	4.5 - 5.0	5.1 - 5.2	5.0	5.1	5.6	5.2	4.4 - 5.2
Multibasic sites	8 - 10	15 – 18	9	6 - 10	ND	3	5
Dislufide-bonded loop	Yes	Yes	No	No	ND	No	No
Calcium binding	Yes	Yes	Yes	ND	ND	No	ND
Thermostability	Yes	Yes	Yes	ND	ND	Yes	Yes
Phosphorylation	Yes	Yes	Yes	ND	ND	Yes	Yes
Sulfation	Yes	Yes	Yes	Yes	ND	Yes	ND
O-glycosylation	Yes	Yes	Yes	ND	ND	ND	Yes
N-glycosylation	Yes	Yes	Yes	No	No	No	No

Table 1: Physico-chemical properties of the members of the Granin family. CgA denotes chromogranin A, CgB chromogranin B, SgII secretogranin II, SgIII secretogranin III, SgIV secretogranin IV, SgV secretogranin V, SgVI secretogranin VI, and ND not determined. † Amino acid residues are for mature protein without signal peptide. ‡ The molecular mass was calculated from the primary structure. The apparent molecular mass was determined with the use of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis. § Multibasic sites refer to sites with two or more consecutive Arg or Lys residues. Table *after* Taupenot *et al.* (27).

1.2.2. Chromogranin A is an on/off switch for granule biogenesis

Chromogranins A and B share a region that includes two cysteine residues in a disulfide bond in their N-terminal section. Chromogranin A (CgA) is considered to be a precursor for various biologically active peptides (28). Several single and paired basic residues are present within its primary amino acid sequence comprising cleavage sites for prohormone convertases. CgA is a ~50 kDa acidic glycoprotein. It is thought to be co-

stored and co-secreted with hormones and neurotransmitters in a variety of tissues like dense core storage vesicles of adrenal medulla and sympathetic nerves (27, 29).

CgA may play a key role in secretory granule biogenesis and hormone packaging in neuroendocrine cells (30, 31). CgA has been proposed to comprise of an "on/off" switch that controls dense-core secretory granule biogenesis (30, 31). This group used antisense RNA to down-regulate CgA and CgB expression in neuroendocrine PC12, pituitary cells and non-endocrine cells, and found that there was a profound loss in dense core secretory granules and hence a reduction in secretory proteins. This defect could be rescued by transfecting bovine CgA into PC12 cells lacking CgA. There was no effect on dense core secretory granule biogenesis when CgB was knocked down. Hence, CgA was proposed to control secretory vesicle biogenesis.

1.2.3. Chromogranin B (or Secretogranin I) plays direct role in secretory granule biogenesis.

Chromogranin B (CgB) is one of the members of the Granin family of proteins that are thought to be co-released with neurotransmitters from neurons and endocrine cells. Like all other Chromogranins, it is extremely heat stable, highly acidic, and post-translationally modified by phosphorylation, sulfation, and glycosylation. Similar to CgA, CgB contains a disulfide-bonded loop near the N-terminus (32, 33), which is thought to be essential for aggregative processing of regulated secretory proteins that are sorted into immature secretory granules (34, 35). The variable internal region of CgB is characterized by glutamic acid clusters, hydrophilicity, and potential dibasic cleavage sites for the generation of smaller, perhaps hormone-like, peptides. In the pancreas, Chromogranin B regulates the secretion of hormones, such as IAPP (islet amyloid polypeptide) and insulin. Chromogranin B is expressed primarily in brain, anterior pituitary, and adrenal gland. Thus, Chromogranin B functions in secretory protein

sorting, while its proteolytic peptide fragments are thought to be involved in hormonal signaling (26, 28, 35, 36).

Like CgA, CgB has been proposed to be a necessary component of the cellular machinery for secretory vesicle biogenesis. CgB has been found to be more effective in the induction of secretory granule biogenesis than CgA. Huh *et al* made a direct comparison of CgB with the similar role of CgA (37).They compared the two proteins in neuroendocrine and non-neuroendocrine cells. They used transfection techniques to show that CgB could induce more granule biogenesis in non-endocrine cells. They exploited siRNA techniques to inhibit the expression of CgB and CgA, and concluded that inhibition of CgB expression was more effective than CgA at suppressing secretory capabilities. Remarkably, they found that CgB was localized to the nucleus and participated in transcription control unlike CgA , thereby stating that CgB played a direct role in secretory granule biogenesis (38, 39). Mechanistically, this finding is of interest because it suggests that the effects of Granins on secretory vesicle biogenesis may be indirect, i.e. through control of transcription of an unknown factor that is itself critical for granule formation. However, at the present time this model is highly controversial.

1.2.4. Secretogranin II – a marker for dense core vesicles.

Secretogranin II (previously called Chromogranin C) is expressed in a wide variety of endocrine and neuronal cells that package and sort regulatory peptides into secretory granules. Secretogranin II have been shown to be less related to Chromogranins in terms of their cDNA sequence, but also have been shown to possess some common features with Chromogranin, such as a similar primary and secondary structures, containing multiple potential dibasic cleavage sites, and the ability to modulate proteolytic processing of enzymes in neuro-endocrine granules (24-26, 34, 40). The SgII gene has been shown to bear the same cAMP response element (CRE) and TATA box motif that is

conserved in CgA and CgB. Similar to other proteins in the same class of the Granins these proteins also have an acidic pI due to the high content of acidic amino acids resulting in a high net negative charge. The acidic feature has also been attributed to phosphorylation on serine and sulfation on tyrosine and O-linked carbohydrates. These proteins have also been shown to be tyrosine sulfated (24-26, 34, 40). SgII has been shown to be a calcium binding protein (41). SgII has been proposed to interact with syntaxin in the chromaffin secretory granules, which share similarities with the secretory mechanisms in mast cells (42). SgII release can be stimulated by various secretagogues such as ionomycin and PMA (phorbol myristate acetate), which produce calcium signals and kinase activation, respectively (43, 44).

SgII has also been thought to participate in secretory granule biogenesis (45). Like CgA and CgB, SgII also undergoes acidic pH and Ca^{2+} -dependent aggregation and also interacts with the secretory granule membrane at the intra-granular pH 5.5. SgII also interacted with the secretory granule membrane at pH 5.5 and also dissociated from it at pH 7.5, like CgA and CgB, implying similar roles of SgII in the cell to those of CgA and CgB (45).

1.2.5. Secretogranin III has an unknown function.

One of the Granins whose function has remained elusive is Secretogranin III. This protein comprises 468 amino acids and like other Granins, is an acidic secretory protein that is expressed in storage vesicles of neuronal and endocrine cells (26, 46, 47). Ultrastructural studies have detected the presence of SgIII in intracellular vesicles (40, 48). It has been shown that SgIII is a sulfated precursor protein and that it is proteolytically processed at two dibasic sites (48). However, Ottiger *et al.* reported that SgIII was not significantly proteolysed and that it functions as a full-length protein, unlike SgI and SgII (46). We will present data that support the findings of Ottiger *et al.*

SgIII binds to CgA and forms a heterophillic complex in secretory granules of corticotrope-derived AtT-20 cells and Insulinoma-derived INS-1 cells. SgIII may be a sorting partner for CgA that targets secretory vesicles in pituitary and pancreatic cells (49-51). Immunocytochemical studies have proved that SgIII co-localizes with CgB in the pituitary gland (49-51). Hosaka *et al.* proposed that both of these Granin proteins might function redundantly during storage and transport of the hormones in the TGN (Trans Golgi Network) and secretory granules of the endocrine cells. Hosaka *et al.* have demonstrated that SgIII is localized to the secretory granules and that it makes a complex with the cholesterol components of the secretory granule membrane (49-51). SgIII also interacts with Carboxypeptidase E (CPE) and therefore promotes pro-hormone sorting within secretory granules. It has been suggested that CgB helps in the sorting of Proopiomelanocortin fragments to the secretory granules in chromaffin cells (36).

1.2.6. Summary

- The Granin Family is a unique group of acidic, soluble secretory proteins comprising of Chromogranin A, B and Secretogranin I-VI.
- Three Granins have been shown to be involved in secretory granule biogenesis in neuroendocrine and neuronal cells.
- Other Granins may not be involved in the biogenesis of granules, but instead have other roles as hormone precursors.
- There has been no published analysis of expression, or function, of Granins in the mast cell system, but their involvement in secretory granule biogenesis in other secretory cell types leads us to hypothesize that Granins may be components of the secretory machinery in mast cells.

Chapter 2. Aim and Objectives

2.1. Aim

We hypothesise that Secretogranin III (SgIII) is a component of the secretory machinery in mast cells.

2.2. Objectives

- To identify the molecular machinery that controls the release of inflammatory mediators from mast cells.
- To investigate if Granins could be a component of the secretory machinery in mast cells.
- To identify if SgIII is present in mast cell granules.
- To investigate if altered expression of SgIII changes the secretory granule properties of mast cells.
- To investigate if SgIII is a necessary component of mast cell secretory vesicles.

Chapter 3. Materials and Methods

3.1. Cell Culture Methods.

3.1.1. Maintenance of cell cultures.

RBL2H3, HEK293 and PC12 cells were maintained in 1X DMEM (Cellgro, VA), 10% fetal bovine serum (Cellgro, VA) and 2mM glutamine (Invitrogen, Carlsbad, CA) in humidified 5% CO_2 at 37°C. PC12 cells were maintained in with 500mM of HEPES (Fisher Scientific, Fair Lawn, New Jersey) and 500mM of Sodium Pyruvate (Sigma Aldrich, St. Louis, MI) buffer in addition to 10% FBS and 2mM glutamine in humidified 5% CO_2 at 37°C.

The stably transfected cell lines were maintained in antibiotics added to the normal media of 1X DMEM, 10% FBS and 2mM glutamine, such as 200 μg/mL zeocin (Invivogen, San Diego, CA) for RBL-SgIII and PC12-SgIII. HEK-SgIII cells were maintained in 5 μg/mL of blasticidin (Invitrogen, Carlsbad, CA) and 200 μg/mL of zeocin.

3.1.2. Production of constitutive/ inducible stable cell lines

The RBL2H3 and PC12 cell lines were electroporated with V5-SgIII construct to produce constitutive expression system. The cells were electroporated with 15 μg of V5-SgIII pcDNA4/TO (2.8 μg/μL) in 500μl volume of 1x DMEM, 10% FBS and 2mM glutamine at 280 KV and 950 μF in a 4 mm path length cuvette (BioRad, Hercules, CA). Clonal cell lines were selected by limiting dilution in 400 μg/mL zeocin only. The clones were screened by immunoprecipitation and Western blotted by Anti-V5.

For production of TRex HEK293 cells with inducible expression of SgIII, parental cells were electroporated with 15 μg of pcDNA4/TO V5-SgIII plasmid DNA in a 500 μL volume of DMEM/10% FBS/2 mM Glutamine. Electroporations were performed at 280

V/950 μF in a cuvette (BioRad, Hercules, CA). Clonal cell lines were selected by limiting dilution in 400 μg/mL zeocin and 5 μg/mL blasticidin, and screened by immunoprecipitation and Western blot. Transfected gene expression was induced using 1 μg/mL tetracycline for 16 h at 37°C.

3.2. Transcriptional array analysis

These experiments were performed prior to the candidate joining Dr. Turner's laboratory. Total RNA was extracted from adherent RBL2H3M1 cells that were either left untreated or exposed to IgE + 100 ng/mL DNP-BSA for the indicated times. Total RNA was purified using a Nucleospin RNA II kit according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA). Prior to hybridization on the BD Atlas™ Rat 1.2 Arrays, total RNA samples were labeled with [α-^{33}P]dATP using the Atlas Pure Total RNA Labeling System (BD Biosciences, Palo Alto, CA). A set of four matched BD Atlas™ Rat 1.2 Array blots were hybridized (68°C, 16 h). Blots were wrapped and exposed to storage phosphor screens (Packard Biosciences, Meridien, CT) for 8 days. Data were captured using a Cyclone System (Packard Biosciences, Meridien, CT). BD Atlas Image 2.7 (BD Biosciences, Palo Alto, CA) software was used to assign genes to specific coordinates on the array membranes, to correct for background counts, to establish the threshold for positive gene expression (2 x background level counts + 10%), and to normalize between-blot hybridization intensity differences based on the median-signal intensity. Per gene differences in mRNA levels were expressed as a fold-change relative to the corresponding unstimulated controls. BD Atlas Navigator 2.0 (BD Biosciences, Palo Alto, CA) software was used to analyze trends within the data sets.

3.3. Protein Biochemistry Methods.

3.3.1. Antibodies.

Antibody	Source	Immunogen	Species recognized
Secretogranin I	Affinity purified rabbit polyclonal (IgG) (Abcam)	C-terminus of CgB conjugated to ovalbumin	Mouse, rat and human
Secretogranin II	Affinity purified goat polyclonal (IgG) (Santa Cruz Biotechnology, Inc.)	C-terminus of SgII of human origin	Mouse, rat and human
Secretogranin III	Affinity purified goat polyclonal (IgG) (Santa Cruz Biotechnology, Inc.)	C-terminus of SgIII of human origin	Mouse, rat and human
Grb2	Mouse Monoclonal (Upstate cell signaling solutions)	N-Terminal SH3 region of Grb2	Mouse, rat, human
V5 tag	Mouse Monoclonal (Serotec)	Peptide sequence - GLPIPAPLLGLAST	Mouse
Serotonin	Rabbit monoclonal (Sigma Aldrich)	Serotonin creatinine sulfate complex conjugated with formaldehyde to BSA.	Rabbit
ECL™ Anti-mouse IgG, HRP-linked whole Antibody	Sheep Polyclonal (Amersham)	Fc portion of murine IgG	Mouse
ECL™ Anti-rabbit IgG, HRP-linked whole Antibody	Donkey Polyclonal (Amersham)	Fc portion of rabbit IgG	Rabbit

Anti-goat IgG, HRP-conjugated	Rabbit Polyclonal (Sigma Aldrich)	Fc portion of goat IgG	Goat
Alexa Fluor 488	Goat anti-rabbit IgG (H+L) (Molecular Probes)	Fc portion of rabbit IgG	Rabbit
Alexa Fluor 568	Rabbit anti-mouse IgG (H+L) (Molecular Probes)	Fc portion of mouse IgG	Mouse

Table 2. List of all the antibodies used.

3.3.2. Stimuli and Inhibitors

Stimulus/ Inhibitor	**Name**	**Source**	**Function**
DNP-BSA	Dinitrophenyl-Bovine serum albumin	Sigma Aldrich	Cross links IgE anti-DNP bound to FcεRI and hence stimulate cellular responses
PMA	Phorbol myristate acetate	Sigma Aldrich	Activates protein kinase C by binding to its regulatory domain and also stimulates serine/threonine kinase activity
Ionomycin		Sigma Aldrich	It is an ionophore that causes calcium influx.
Tunicamycin		Sigma Aldrich	Inhibits glycosylation. It inhibits the synthesis of N-

			linked oligosaccharide chains (at an early stage of N-glycosylation) that results in the production of glycoproteins containing missing or altered chains.
Sodium chlorate		J. T. Baker	Inhibits tyrosine directed protein sulfation
Genistein		Sigma Aldrich	Inhibits a range of protein tyrosine kinases and thus down regulates tyrosine directed phosphorylation events/signaling cascades.

Table 3. List of all the stimuli and inhibitors used in the experiments presented.

3.3.3. Transient transfection

HEK293 cells were seeded and grown until 50% confluent. All cDNA was purified using a QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA). The cDNA quality was evaluated using a spectrophotometer and used in transfection only if O.D. 260/280 > 1.7. HEK293 cells were transiently transfected using reagent LT1 (Mirus, Madison, WI). Serum free DMEM (100 µL) and LT1 reagent (10 µL) were added together and vortexed for 2 min. The mixture was then left at room temperature for 15 min. After 15 min, 7.5 µg of V5-SgIII cDNA was added to the mixture and mixed gently. After 15 min at RT, the mixture

was added to the cells in a drop-wise manner while swirling the plate. The cells were then placed in a humidified 5% CO_2 incubator for 48-72 h. The cells were then harvested and lysed as described below.

3.3.4. Electroporation of V5-SgIII cDNA

The cells were suspended in 500 μL of respective media (1x DMEM, 10% FBS and 2 mM glutamine) with no antibiotics. The cell suspension was electroporated with 15 μg of V5-SgIII pcDNA (2.8 μg/μL) at 280 kV and 950 μF in a cuvette. The cells were then plated overnight at 37°C. The electroporated cells were finally trypsinized and resuspended in 20 mL of media with specific antibiotics and then plated onto tissue culture dishes or 96-well plates using the serial dilution technique (Figure 2).

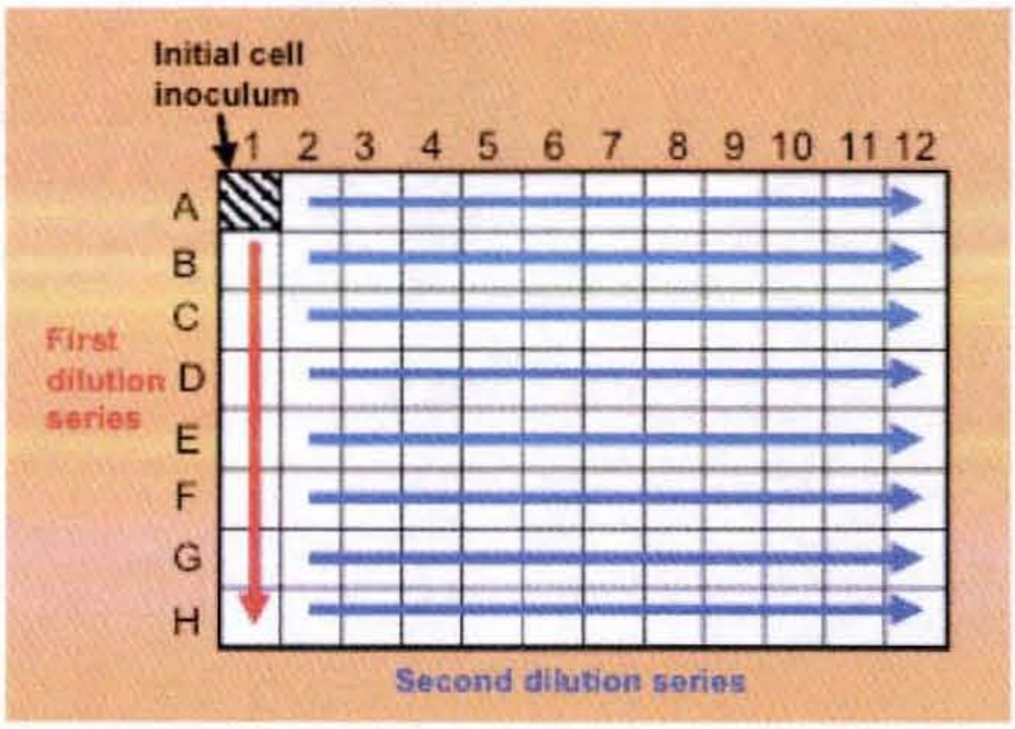


Figure 2. Serial dilution technique.

The cell inoculum (200 μL of $2x10^5$ cells/mL) was placed in the top left well (A1). All wells in the 96-well plates were filled with 100 μL medium except A1. 100μl of cell inoculum was pipetted into B1. The inoculum was mixed well and the 100 μL was pipetted from B1 to C1 and so on till H1 had 200 μL of the cell suspension in media (red arrows). 100μl from H1 was discarded, so that all wells A1-H1 had 100 μL. Using an 8 channel pipettor an additional volume of 100 μL was added in each well of the first column and then 100 μl of cell suspension from the column 1 was pipetted out and transferred entirely to column 2. The cells were mixed well and then the process was repeated across the entire plate (A12 through H12) using the same tip (blue arrows). 100 μL left from the last column was again discarded. The final volume of all wells was brought up to 200 μL and the plate was placed into an incubator at 37°C. Highest cell densities occurred immediately in the wells surrounding A1. Figure *after* Corning single cell cloning by serial dilution manual.

3.3.5. Picking and expansion of selected colonies

After repeated feeding of the colonies with media containing no antibiotics, monoclonal colonies were picked from wells once the colony was ~ 100-200 cells from the 96-well plates. The colonies were pipetted up and down slowly to dislodge them from the surface and transferred into 25 cm^2 tissue culture flasks and subsequently to 150 cm^2 tissue culture flasks for expansion of clonal transfected cell lines with respective antibiotic resistance. After expansion, cell lines were screened for the presence of transfected protein by immunoprecipitation and Western blotting.

3.3.6. Immunoprecipitation and production of IGEPAL lysates

Cells were pelleted (2000 *g*, 2 min) and washed once in PBS at room temperature. Approximately 10^7 cells were lysed on ice for 20 min in 500 µL of 2X lysis buffer (100 mM Hepes pH 7.4, 150 mM NaCl, 80 mM NaF, 20 mM iodoacetamide, 50 mM PMSF (phenyl methyl sulfonyl fluoride), 500 mg/mL aprotinin, 1.0 mg/mL leupeptin and 2.0 mg/mL chymostatin) dissolved in distilled water. Lysates were then clarified (10000 *g*, 5 min at 4°C) so as to avoid the nuclei pellet. For preparation of total protein, lysates were acetone precipitated and again centrifuged at 10000*g* for 5 min at 4°C. For immunoprecipitation, supernatants were tumbled (4°C / 2 h) with the indicated antibody, followed by capture of immuno-complexes using ~50 µg Protein A conjugated to agarose beads (Sigma Aldrich). Beads were dried with Hamilton syringe. Samples were then boiled (8 min) in reducing sample buffer (20% (v/v) glycerol, 62.5 mM Tris-HCl pH 6.8, 0.05% (w/v) bromophenol blue, 2 mM 2-mercapto-ethanol) and then resolved by SDS-PAGE overnight.

3.3.7. Western Blots

After being resolved on SDS-PAGE, the proteins were electro-transferred to Polyvinylidene Fluoride (PVDF) membrane (1.4 A for 180 min in 25 mM Tris base-HCl, 192 mM glycine, pH 8.63). Membranes were blocked using 5% (w/v) non-fat milk (1 h at RT). The membranes were incubated with primary antibodies (antibody dissolved in antibody diluent – TBS, 0.05% TWEEN, 0.05% NaN_3; and 0.05% NaH_3 and 5% BSA) for 16 h at 4°C. The membranes were subsequently washed with TTBS (4 x 5 min) and incubated again with secondary antibodies (HRP-conjugate) for 1 h. The membranes were then again washed with TTBS (4 x 5 min) and then incubated with ECL Plus Western blotting Detection Reagent (Amersham Biosciences, Piskataway, NJ) that helps in detecting the immobilized specific antigens conjugated to the HRP labeled antibodies by producing a high intensity chemiluminescence. The emitted light was then detected using autoradiography film (Kodak Scientific Imaging Films).

3.3.8. Stripping Polyvinylidene Fluoride (PVDF) membrane.

The PVDF membranes after being exposed to ECL Plus Western blot detection reagent was stripped to be re-probed with another antibody. Stripping means the complete removal of the primary and secondary antibody from the membrane. For this, the membrane was first activated with methanol and washed with distilled water and then submerged into stripping buffer (98% (v/v) 2-mercaptoethanol, 20% (v/v) SDS, 1M Tris-HCl, all dissolved in distilled water, pH 6.7). The membranes with the buffer were incubated at 50°C for 30 min with occasional agitation in a water bath. The membranes were washed with TTBS (2 x 10 min) at room temperature. The membranes were consequently blocked and Western blotted.

3.3.9. Protein determination assay.

Protein samples were matched for total protein levels on the basis of a colorimetric protein determination assay. We used the D_c Protein Determination Kit™ from BioRad. After cell lysis, triplicate samples of 1 μL, 3 μL and 5 μL were removed from each lysate. These were transferred to a 96-well micro-titre plate and incubated with 25 μL per well of reagent A' (mixture of reagent A and reagent S), according to the Manufacturer's instructions. At this point 200 μL of reagent B (substrate) was added to each well and the plate was incubated at RT for 10 min. Color development was read at 710 nm in a Benchmark Plus (BioRad, Hercules, CA) plate reader. The triplicates were averaged and a series of normalization ratios were calculated and used in the loading scheme for the SDS-PAGE gels. A comparison of the data from the 1 μL, 3 μL and 5 μL samples ensured that the D_c assay was in its linear range.

3.4. Functional assays for mast cell activation.

3.4.1. Tritiated serotonin release assay.

Adherent RBL2H3 and RBL-SgIII (11.25×10^5 cells/cm^2) were incubated with 1 μg/mL IgE – Anti DNP and 0.5 μCi/mL ^{3}H-serotonin for 16 h at 37°C. Monolayers were then washed with normal media at 37°C (2X) and cells were incubated with 10 μL of indicated stimuli (1 μM PMA, 10 μM ionomycin or 10% triton) or vehicle (PBS) in 190 μL/cm^2 media for 1 h at 37°C. Reactions were stopped on ice. The supernatant was centrifuged (10,000 *g* for 30 sec) and 100 μL of supernatant were counted in liquid scintillation cocktail (Scintiverse BD cocktail, Fisher, Fair Lawn, NJ).

3.5. Microscopy.

3.5.1. Immunofluorescence.

Cells were seeded on 24-well plates with glass cover-slips (4.7 x 10^7 cells/mL). The cells were first fixed with pure methanol at –20°C for 5 min. They were then washed with phosphate-buffered saline (PBS), and then blocked with 0.7% (w/v) Fish skin gelatin (prepared in PBS) for 20 min. The cells were incubated with primary antibody diluted in the blocking reagent (0.7% (v/v) fish skin gelatin) for 1 h. The cells were washed 4 times with PBS and the incubated with Alexa secondary antibodies prepared in the blocking reagent for 20 min. The cells on the cover-slips were washed 4 times with PBS and then finally the cover-slips were dipped in water and mounted on the microscope slides using Crystal mount (Biomeda Corp. Foster City, CA). The slides were then observed 6 h later under confocal microscope and pictures were taken. Imaging was performed with an Olympus IX70 fluorescence inverted microscope with quadruple dichroic filter block and excitation filter set 88000 (Chroma, Rockingham, VT), connected to an F-view monochrome CCD camera.

3.6. RNA Interference

We performed RNA interference experiments with Silencer siRNA Transfection II Kit (Ambion Inc., Austin, Texas). This kit had siPORT Amine Transfection Agent (a proprietary blend of polyamines), GAPDH siRNA as positive control, and a negative control siRNA. This kit is primarily intended for transfection of adherent cells. The transfection agent forms complexes with siRNA facilitating the transfer into cells. Control siRNA is a scrambled sequence that bears no homology to the human, rat or mouse genomes. RBL2H3, HEK293 and PC12 cells were trypsinized or passaged without trypsin as in PC12 cells. The trypsin was inactivated by resuspending the cells in normal

growth medium to reach a concentration of 1 x 10^5 cells/mL. RNA-Transfection agent complex (240 μL per well) was prepared in serum free media. The complex was dispensed into the empty wells of plates (6-well plates) and then cells transferred to the culture plate containing the complex making the total volume to 3 mL per well. The cells were gently mixed with the RNA-Transfection agent complex and incubated for 72 h. All experiments were done under RNase free conditions.

Chapter 4. Results I: Secretogranin III is expressed in mast cells.

4.1. Introduction.

Mast cells are central effectors of immediate and late phase inflammatory reactions. These cells contain numerous electron-dense cytoplasmic secretory granules that store potent inflammatory mediators and are central to mast cell pro-inflammatory functions. On encountering specific antigen, IgE-sensitized mast cells undergo degranulation of their secretory granules, releasing histamine, serotonin, neutral proteases. It has been shown that mast cell activation is a time-dependent process that starts with a stimulatory event accompanied with degranulation in the first 30 min of stimulation. During this time, the mast cell releases its secretory granule contents resulting in the depletion of its levels of proteases, histamine, serotonin and other granule components. This event is followed by a 3-6 h refractory phase when the mast cells are engaged in replenishing their granule content. Approximately 6-9 h after stimulation, the granule content levels are restored and attain the pre-stimulatory levels. The mast cell is then prepared for degranulation in response to new stimulus.

In this Chapter we will present data showing that SgIII is a component of the secretory machinery in mast cells, and that this protein is abundant in mast cells. Moreover, we will also show that SgIII protein levels are regulated in a manner consistent with mast cell activation when cross-linked with FcεRI.

4.2. Results.

4.2.1. Hunting for granule component genes

Prior to my joining her laboratory, Dr. Turner undertook analysis of the genes that are regulated in response to FcεRI activation of mast cells. The assay system chosen was the BD Clontech Rat 1.2 transcriptional array. Transcriptional arrays allow investigators to

map the expression profile of genes of interest in comparison to other related genes immobilized on the array membranes.

Dr. Turner designed and performed a kinetic experiment, looking at changes in gene expression at 0.5 h, 3 h and 9 h after FcεRI cross-linking. These time-points were chosen to represent acute, ongoing and potentially long term alterations in the mast cell transcriptional program. Over time Dr. Turner has performed several analyses of the data set, which comprises normalized transcript levels for 1100 genes at three distinct time-points. One of these analyses sought to identify genes that might be components of mast cell secretory granule. The rationale here is that: Mast cells secrete their granules over a time course of 2-30 min after stimulation. After secretion, there is a well–established refractory phase, during which the mast cells replenish their granule population and re-synthesize the mediators which have been secreted into the extra-cellular milieu. Physiologically, these cells recover and are fully replenished by 6-9 h after the initial secretory event. We reasoned that searching for genes whose transcript levels were upregulated following secretion, but then returned to baseline, could identify novel components of mast cell secretory machinery. Figure 3 (left panel) suggests that this is a valid concept. Here, we mined the data set for genes with a profile of bell-shaped regulation following FcεRI cross-linking. Interestingly, one prominent cluster that appeared in this output included the genes for several mast cell secreted proteinases, which are intra-granular components. For comparison, Figure 3 (right panel) shows example profiles of genes which are either not significantly regulated, or in which a sustained elevation in transcript levels is observed. These are genes that we would not propose to be components of the secretory machinery. We were intrigued to note that the transcript levels for Secretogranin III, which is not a secreted proteinase of the mast cell, were also following the bell-shaped expression pattern. These data raised the question of whether SgIII could be a component of the mast cell secretory machinery.

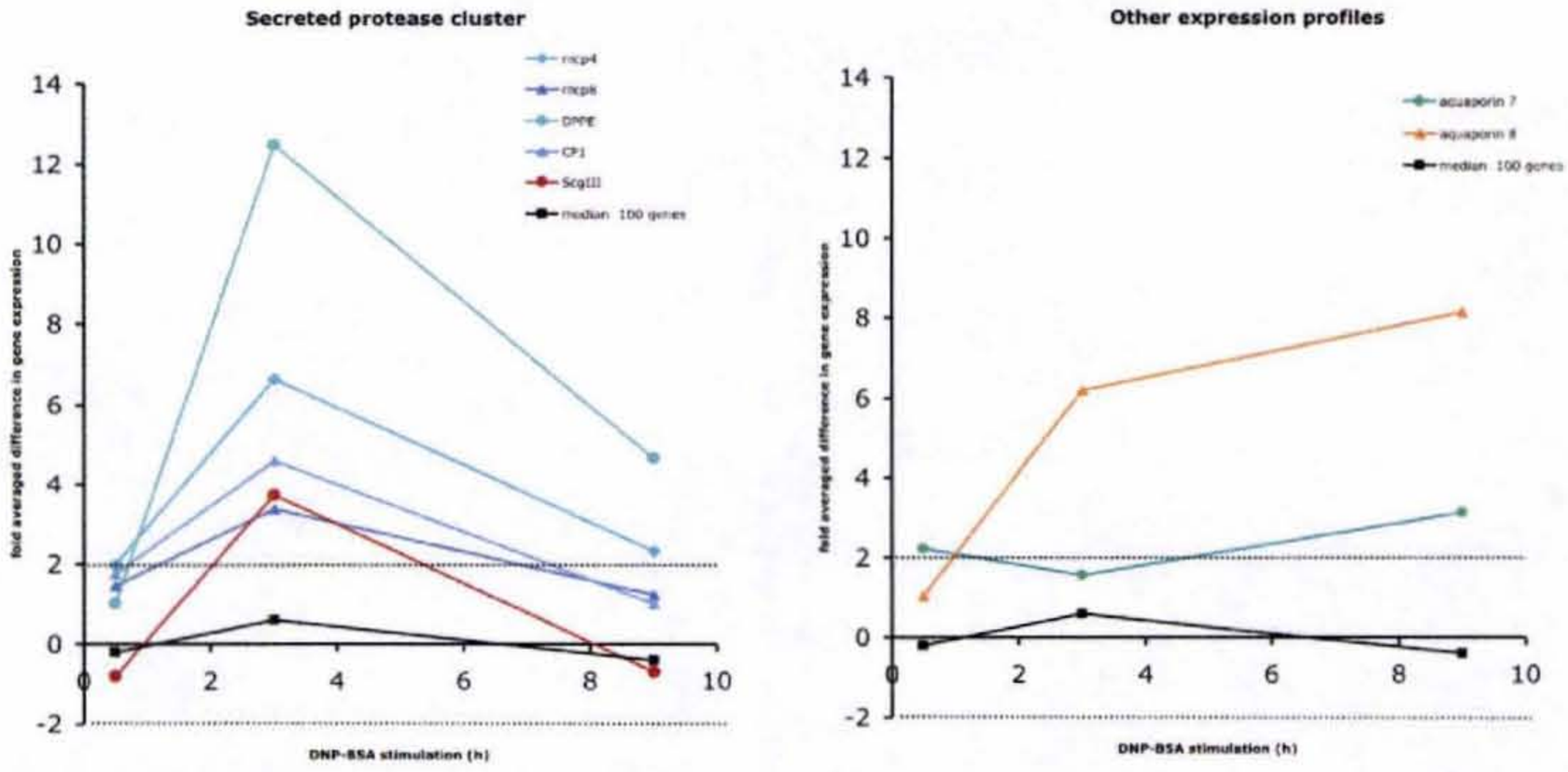


Figure 3. Hunting for granule component genes.

Left panel shows the expression pattern of genes of mast cell secreted proteinases with a transient transcriptional up-regulation at 3 h . Right panel shows the expression patterns of other genes that did not show any significant similarity in expression profile to that of mast cell proteases.

4.2.2. Expression analysis of Granins in mast cells.

Our initial literature survey suggested that the Granin are an extensive protein family that have roles in vesicle trafficking. We used the array data set described above to ask whether transcripts for the Granin family are extensively represented in mast cells, and whether any of these genes appear to be regulated in the same fashion as SgIII.

Table 4 suggests that transcripts for the Granins SgI, SgII, SgIII and SgV are present in mast cells at levels above background hybridization. However, it is noteable that only transcripts for SgIII show the distinctive expression profile described above. Having confirmed by Western blot (Figure 4) that SgIII is present in a variety of mast cell lines, we decided to focus on this enigmatic protein for the rest of the project. The data (Figure 4) show that Secretogranin III is expressed in RBL2H3 (mucosal mast cells) mastocytoma P815 cells, epithelial HEK cells and primary bone marrow derived mast C-57 cells, and that the SgIII protein is abundant in each of the mast cell line tested (RBL,

P815, C-57). We also noted that SgIII migrated as a single species on SDS-PAGE (Figure 4), supporting the data of Ottiger *et al* who suggested that SgIII is not proteolysed to any bioactive peptides in a similar manner to other Granin family members and that it functions as a full-length protein (46).

Gene	**Positive mRNA expression in mast cells**	**Average Difference Ratio**		
		0.5h/oh	**3h/oh**	**9h/oh**
SgI	+	No change	–	No change
SgII	+	–	–	–
SgIII	+++	No change	++	No change
SgV	+	No change	No change	No change

Table 4. Table summarizing the expression properties of Granin family members in RBL2H3 mast cells. Data are derived from transcriptional array analysis as described in Methods. Positive mRNA expression is defined as 2-fold increased signals over averaged background intensity. Time course data refer to fold change in transcript level with 0.5 h, 3 h or 9 h exposure to FcεRI stimulation.

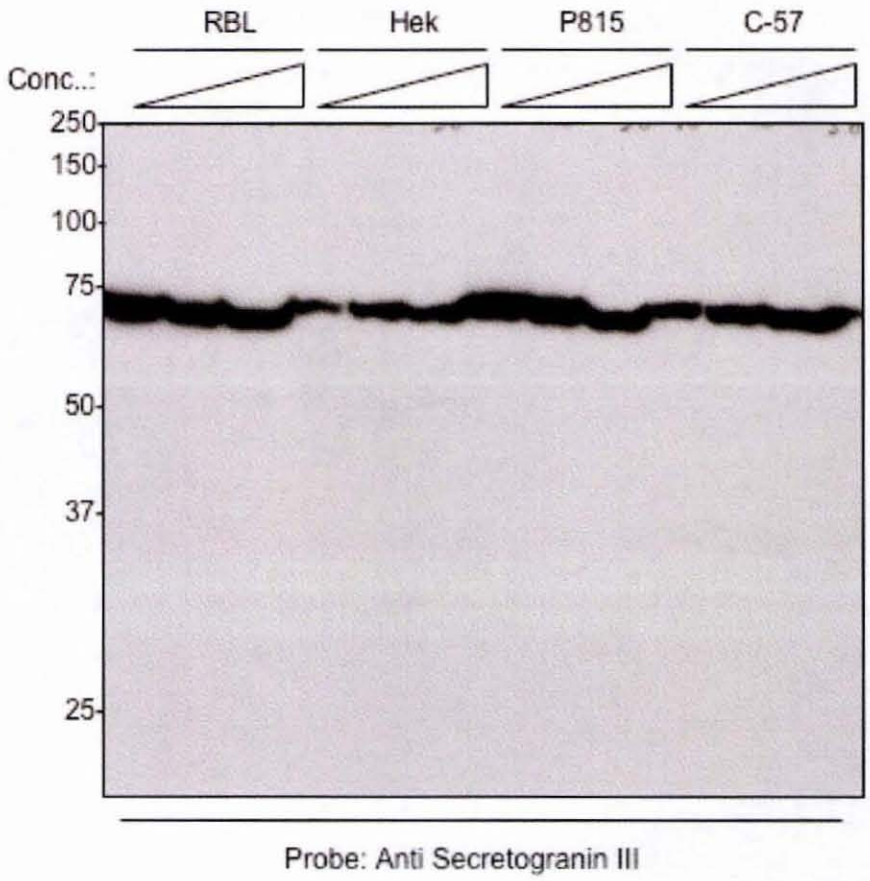


Figure 4. SgIII protein levels in mast cells.

IGEPAL lysates were prepared from $4x10^5$ cells/mL, $8x10^5$ cells/mL and $2x10^6$ cells/mL RBL2H3 (rat mast cell), $4x10^5$ cells/mL, $8x10^5$ cells/mL and $2x10^6$ cells/mL HEK (human endothelial cells) $2.14x10^6$ cells/mL, $4.28x10^6$ cells/mL and $10.72x10^6$ cells/mL P815 (mouse mast cell), or $2x10^6$ cells/mL, $4x10^6$ cells/mL and $10x10^6$ cells/mL C-57 murine bone marrow derived mast cells. Total protein was recovered by acetone precipitation and was resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 µg/mL Anti-SgIII. MW shown in kDa. Predicted MW of SgIII is 68 kDa.

4.2.3 Protein expression of SgIII in mast cells following pharmacological activation.

Our mRNA data suggested that SgIII transcript levels increased after induction of secretion by FcεRI cross-linking. This expression profile mimicked that of the secreted mast cell proteases, leading us to ask whether this pattern would be observed in SgIII protein levels as well. Figure 5 shows that SgIII protein levels were reduced following

stimulation of mast cells to induce degranulation and were later restored. RBL2H3 cells were stimulated with PMA (which activates protein kinase C) and ionomycin (which causes calcium influx). The combined application of these stimuli results in mast cell activation, mimicking the signaling events that follow FcεRI cross-linking. To study the expression of SgIII at the protein level, we used an antibody directed against SgIII to probe the RBL cell lysates by Western blot analysis. Our results show that resting cells express SgIII protein, which is markedly depleted at 60 min and 120 min after stimulation. SgIII protein starts to reappear at 4 h after stimulation and this expression increases at 24 h after stimulation, and now resembles the protein expression at resting state. The explanation for this could be that contents of the granules are released in the first 2 h followed by the initiation of replenishment of the mediators in the granules at 4 h and finally 24 h after stimulations. These data seem to be consistent with the possibility that SgIII is released from mast cells during degranulation and is then replenished like other granule components. An alternate possibility is that FcεRI stimulation causes specific degradation of SgIII. These two possible explanations will be resolved in later experiments.

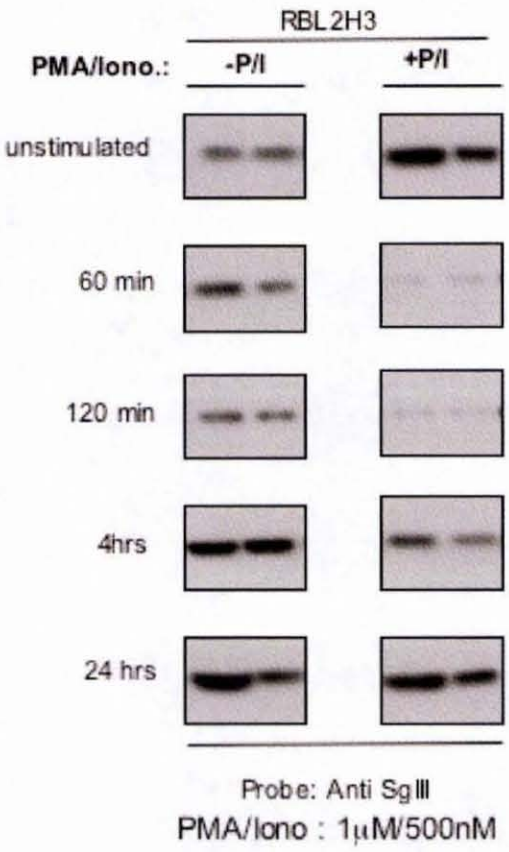


Figure 5. Western blot analysis of SgIII in mast cells following pharmacological activation.

RBL2H3 mast cells were stimulated with PMA and ionomycin at the following concentrations for 0, 60 min, 120 min, 4 h and 24 hrs. After stimulation, cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 μg/mL Anti-SgIII.

4.3. Discussion.

One of the goals of Dr. Turner's lab is to identify genes that encode components of the mast cell secretory machinery. In this chapter we mined for genes that are regulated in response to FcεRI activation of mast cells. Specifically, transcriptional array analysis was done to identify genes that showed a transient transcriptional up-regulation following the FcεRI cross-linking. The rationale for seeking such genes was that mast cell secretes their granule over a time course of 2-30 min after stimulation. Mast cells later replenish their granule population restoring the mediators in them. We observed that the genes of

mast cell secreted proteinases showed specific transient transcriptional up-regulation at 3 h. Interestingly, SgIII followed the same expression profile as mast cell secreted proteases with a maximum transcriptional up-regulation at 3 h after stimulation. Other Secretogranins tested such as, SgI, SgII, SgV did not follow the bell shaped expression profile. This suggested that SgIII could be unique among its family members as it bears a resemblance to mast cell secreted proteinases at mRNA levels Studies at mRNA level needed to be expanded to the study of SgIII protein levels in order to confirm that alterations in mRNA are directly translated to change in protein levels. Western Blot analysis was performed to test the presence of SgIII in mast cells. The analysis showed that SgIII was abundantly present in mast cells (RBL2H3, P815 and C-57). Pharmacological stimulation of mast cells, caused depletion in SgIII protein levels at 60 min and 120 min, which were later restored (at 4 h of stimulation). There could be two possibilities to explain this particular protein expression pattern of SgIII in stimulated mast cells. First, it is possible that SgIII is being degraded at the 60 min and 120 min time intervals after stimulation and later being reformed or reconstituted. There is also a possibility of SgIII being secreted in the same manner as mast cell proteases. If this option is true then the appearance of secreted SgIII into the extra-cellular media should be detectable. We shall perform experiments in the later chapters to ask if the secreted protein appears in the extra-cellular media or is it completely lost in a cytosolic protein degradation pathway.

With the idea in mind that SgIII could be a component of the mast cell granule, we asked whether increases in the cellular levels of SgIII could cause any alteration in the granule content of mast cells. For this we designed an over-expression system, which is dealt in the next chapter.

Chapter 5. Results II: Development of an over-expression system for SgIII.

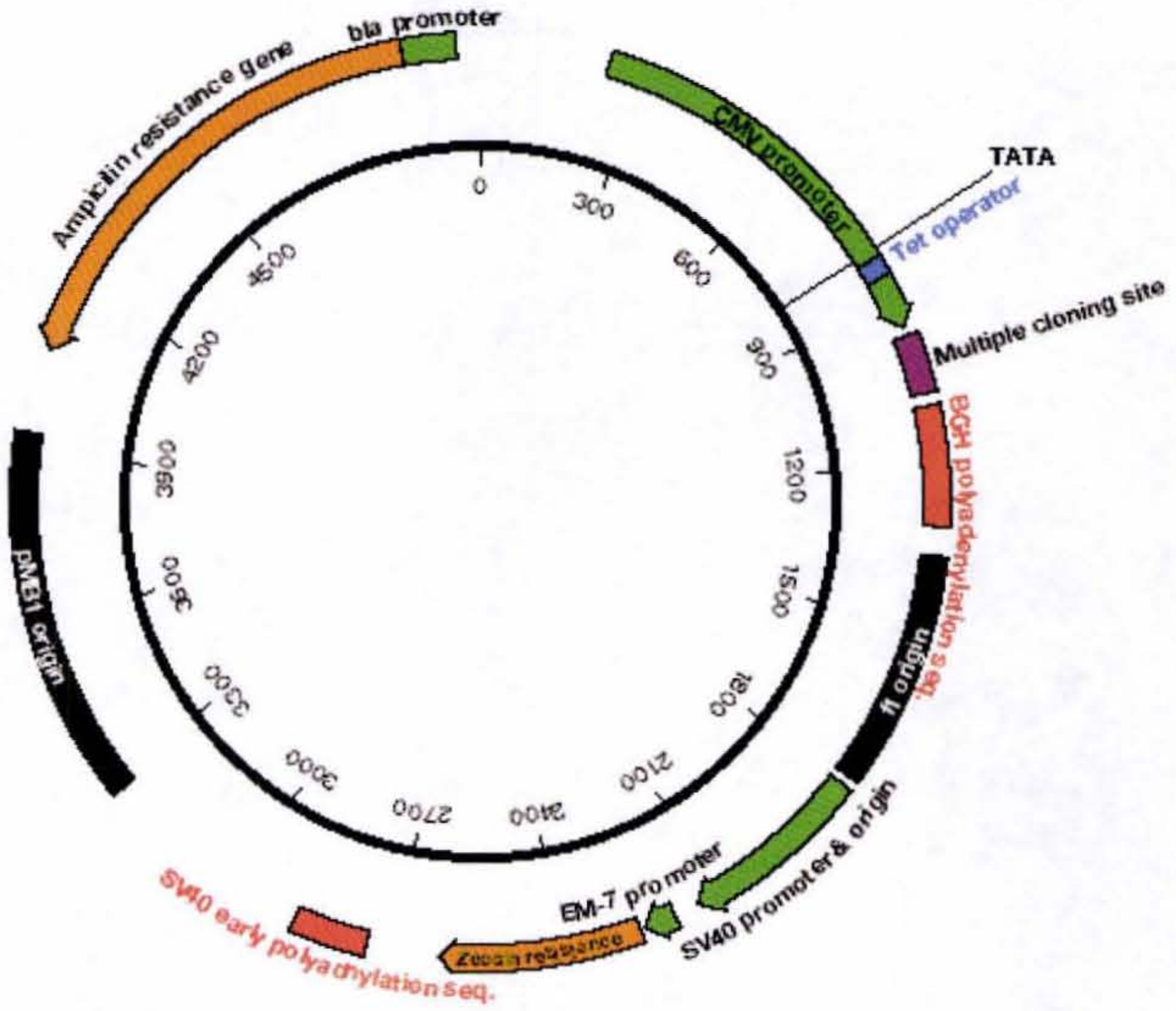
5.1. Introduction.

We hypothesized that SgIII may be a component of mast cell secretory vesicles. RNA (micro-array analysis) and protein analysis (using Western blotting with antibody that is specific to SgIII) have confirmed that this protein is actually expressed in mast cells. Moreover, we also found that SgIII is depleted from mast cells during the time-course of the secretory response. Our next approach was to design an expression system to ask whether over production of SgIII caused alteration in the secretory machinery of the mast cells.

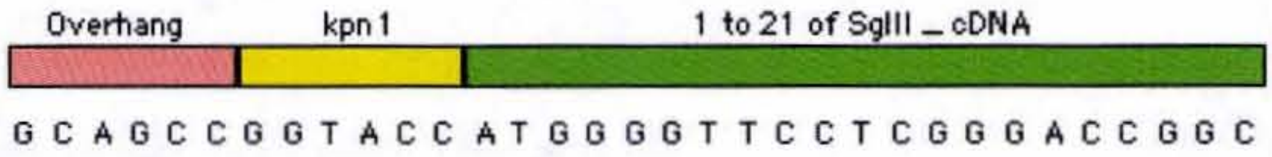
We designed over-expression systems in RBL2H3 rat mast cells, HEK293T-REx™ human endothelial cells and PC12 rat adrenal pheochromocytoma cells. RBL2H3 represents mast cells. HEK cells were chosen as they are highly transfectable cellular expression system with no secretory characteristics. PC12 represent neuroendocrine cells where Granins have previously been shown to control secretory events.

Prior to my joining the lab the sub-cloning of SgIII cDNA to pcDNA4/TO vector with a V5 epitope tag on the C-terminus had been completed (Figure 6). This epitope tag, (the sequence is GLPIPAPLLGLAST), facilitates detection of the over-expressed protein by Western blot and immunohistochemistry. Since the construct had been made our next step was to verify whether the construct could produce a full-length protein of the desired molecular weight.

A)



B) i.



ii.

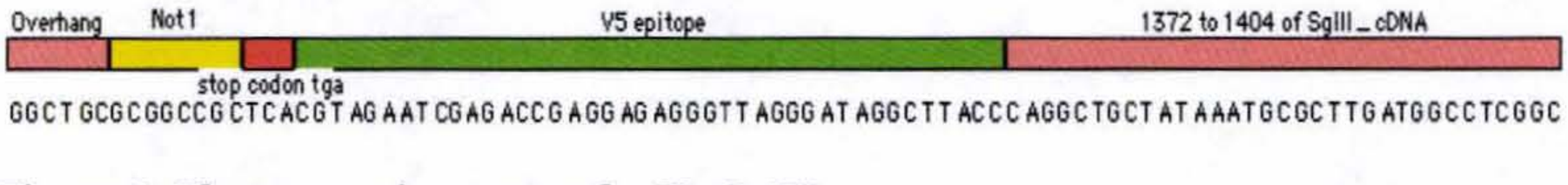


Figure 6. The expression vector for V5-SgIII.

A. The pcDNA4/TO plasmid. This plasmid contains the control elements from the bacterial tetracycline resistance operon to effectively repress, and de-repress, transcription of genes cloned into the multiple cloning site. When stably integrated into the target cell line, which already contains the genomically integrated pcDNA6/TR, repression of the gene of interest can be alleviated with the addition of tetracycline (1mg/mL 16 h).The mammalian drug resistance gene for zeocin allows for effect selection of genomic integration. The ampicillin resistance gene allows for selection of *E.coli* containing the plasmid at a reasonable copy number to survive the ampicillin selection. **B. Primers for the sub-cloning**. These primers (i. and ii) were used to PCR, then subclone SgIII with the addition of a V5 epitope tag, at the extreme carboxyl-terminus. The SgIII cDNA was sub-cloned to pcDNA4/TO vector with a V5 epitope tag at the C terminus. After digestion with KpnI and NotI the SgIII construct was ligated into the pcDNA4/TO vector and the construct was verified by sequencing. This sub-cloning was performed by Dr. A. Stokes.

5.2. Results

5.2.1. Transient transfection of the V5-SgIII construct

An over-expression system for SgIII was designed to ask if the presence of protein caused any alteration in the secretory machinery of the mast cells. The rat SgIII cDNA was sub-cloned into the pcDNA4/TO vector (Invitrogen, Carlsbad, CA) and verified by sequencing. During this cloning a V5 epitope tag was added at the C-terminus to facilitate detection in Western blot and immunocytochemistry. The construct was tested in transient transfection to verify if it produced a full-length protein (Figure 7). HEK cells were transiently transfected using lipid mediated transfection method. After 48 h of transfection, cell lysates were prepared and then immunoprecipitated with Anti-V5 to detect the protein expression (Figure 7, right panel). For comparison, we prepared IGEPAL lysates of untransfected HEK cells in a similar way. Immuno-complexes were then resolved and Western blotted similar to transfected cells (Figure 7, left panel). We observed the presence of V5 expression in the transfected, but not control cells, suggesting that the construct was good and could produce full-length protein after transfection into host cells.

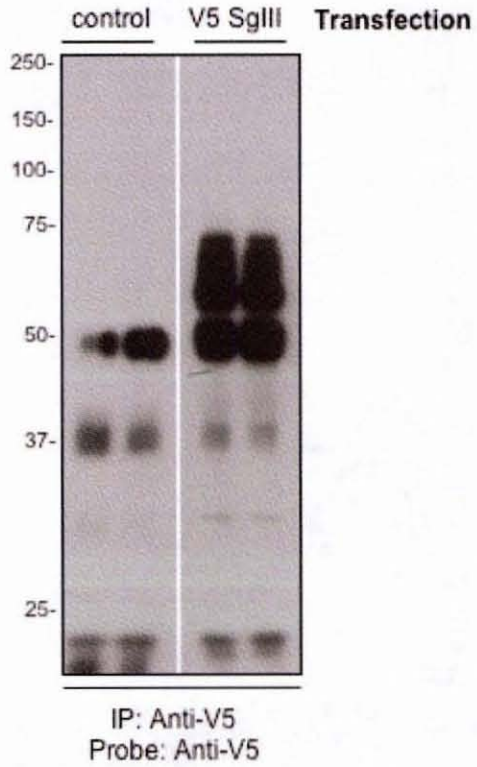


Figure 7. pcDNA 4/TO-V5-SgIII encodes a full-length protein.

HEK cells were transfected with the pcDNA4TO-V5-SgIII construct using the Mirus LT2 reagent, or left untransfected (control). After 48 h for protein expression, cell lysates were prepared and immunoprecipitated using 2 µg Anti-V5. Immunocomplexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. MW shown in kDa.

5.2.2. Production of stable cell lines with over-expression system in RBL2H3 mast cells.

The SgIII-V5 construct had the capability to produce full-length SgIII-V5 protein upon transient transfection. The construct was then electroporated into RBL2H3 mast cells for the production of an over-expression system. An over-expression system could enable us to study the effects of over-production of SgIII protein on the granule content in mast cells. Clonal cell lines with over-expression of SgIII were selected by limiting dilution in zeocin-containing media, and screened for expression of V5-SgIII by immunoprecipitation and Western blot using Anti-V5 for the presence of V5-reactive protein. We obtained several positive clones expressing V5-SgIII (Figure 8).

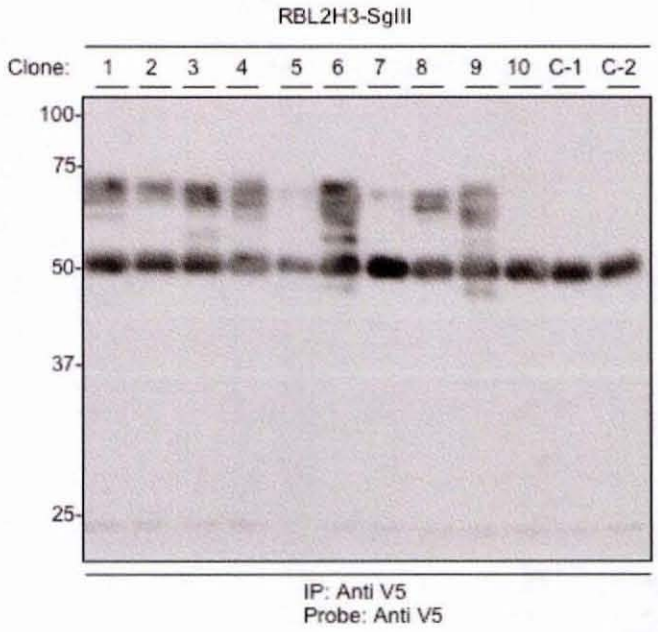


Figure 8. Stable cell lines of RBL2H3 with over-expression of SgIII.
RBL2H3 transfected with the pcDNA4TO-V5-SgIII construct using electroporation, and clonal cell lines were derived by serial dilution and selected in zeocin media within 14 days of transfection. Cell lysates from control cells and transfectants were prepared and immunoprecipitated using 2 µg Anti-V5. Immunocomplexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. C-1 and C-2 are control RBL2H3 cells, that are not electroporated and do not express V5-SgIII. MW shown in kDa.

5.2.3. Over-expression of V5-SgIII in HEK epithelial cells.

5.2.3.1. Production of stable cell lines over-expressing V5-SgIII in HEK

We chose an inducible system, in order to have control over when, and how much, exogenous SgIII protein is produced. The T-REx™ System allows high levels of inducible protein expression in mammalian cells that constitutively express the tetracycline repressor protein. The HEK293T- REx™ cell line is a permanent line of primary human embryonic kidney. The HEK293T-REx™ cell Line stably expresses the tetracycline repressor protein (Invitrogen, Carlsbad, CA). T-REx™-based cell lines exhibit low basal expression of the protein of interest in the repressed state and high expression upon

treatment with tetracycline. The clonal cell lines thus obtained by electroporation were incubated in presence and absence of tetracycline (Figure 9a). The lysates obtained were immunoprecipitated and Western blotted consequently for the presence of Anti-V5 reactive protein. We were again successful in getting positive clones that expressed the protein with desired molecular weight.

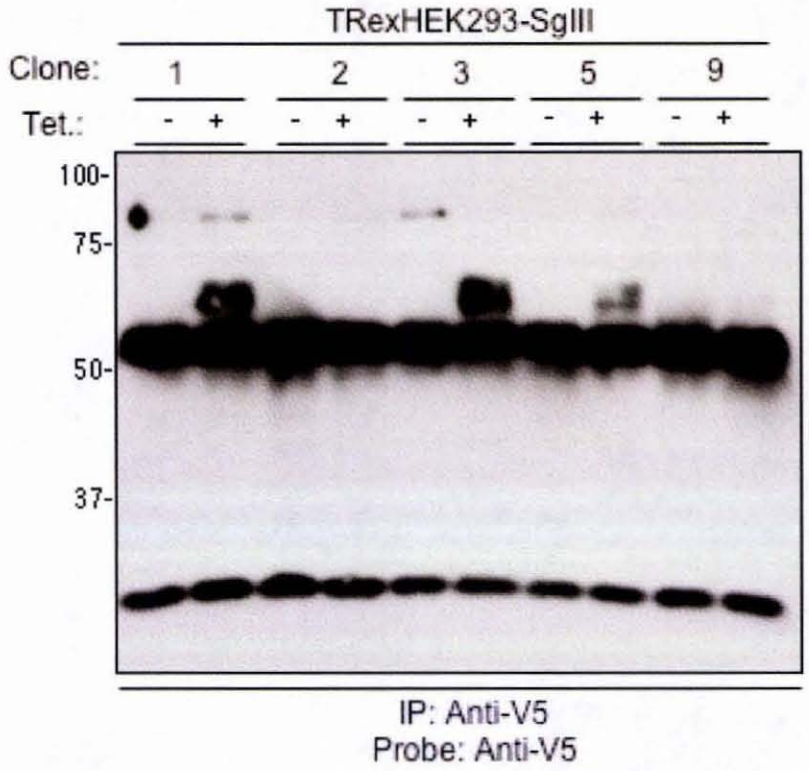


Figure 9a. TRexHEK293 with stable over-expression of SgIII.

T-REx™ HEK293 cells were transfected with the pcDNA4TO-V5-SgIII construct using electroporation, and clonal cell lines were derived by limiting dilution and selected in zeocin/blasticidin media within 14 days of transfection. Cell lysates from control cells and transfectants in presence and absence of tetracycline (1 μg/μL for 16 h) were prepared and immunoprecipitated using 2 μg Anti-V5. Immunocomplexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. MW shown in kDa.

5.2.3.2. Kinetics of the over-expression of the protein

The tetracycline inducible system allows us to control the time-course and intensity of protein expression. We assayed kinetics of V5-SgIII production after tetracycline induction in order to establish the time-course of expression with tetracycline that gave

optimal protein production. Optimal here is defined as production of non-saturating amounts of fully processed protein. The selected clone of HEK-SgIII (#1) was incubated in the presence and absence of tetracycline to study the kinetics of protein production. Our data suggests that V5-SgIII expression is initiated after 2 h of tetracycline stimulation and the expression keeps on increasing over 2-16 h, at which point a plateau is reached and there is no further increase in protein expression. Absence of any low molecular weight forms at any time points, suggested that V5-SgIII protein, was not proteolysed. This observation was again consistent with Ottiger *et al.* who stated that SgIII is not proteolysed like other Granin members and is always produced as a full-length protein instead of being cleaved at any point (46). At the 16 h time point the protein is heavily expressed (Figure 9b). No further increase in protein expression was seen after 16 h. The membrane was stripped and re-probed with Anti-SgIII to show that the V5-reactive protein was indeed SgIII (Figure 9c). We noticed that the Anti-SgIII Western blot was much fainter than the Anti-V5 Western blot. This may indicate that the SgIII Antibody is of a lower affinity. Although, it works well to identify SDS-denatured protein in total lysates, it has significant limitations when visualizing the protein derived from Anti-V5 immunoprecipitation or in immunofluorescence applications. The fact that the Antibody is poor at recognizing native SgIII (in immunoprecipitation and immunofluorescence), it suggests that this is simply a low affinity antisera, rather than there being a specific problem with the structure and the post-translational modifications of SgIII-V5 that interferes with Anti-SgIII recognition. Also, the goat secondary antibody used for Anti-SgIII means that it is not possible to directly compare intensities with the Anti-V5 Western blot that are visualized with mouse secondary antibody.

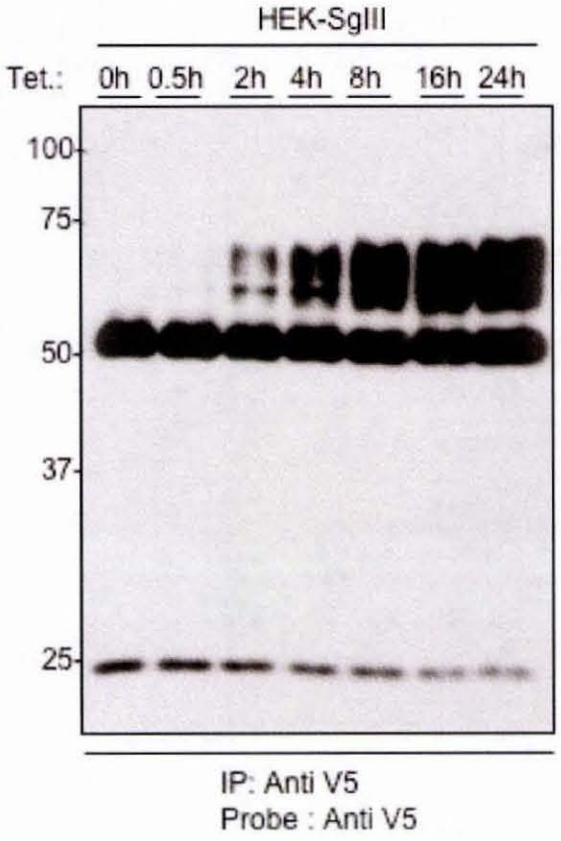


Figure 9b. Kinetics of SgIII over-expression.

HEK-SgIII cells were induced with tetracycline (1µg/mL) and cells were harvested at different time points (0.5 h, 2 h, 4 h, 8 h, 16 h and 24 h). Cell lysates (500µg) were prepared from V5-SgIII expressing cells and subjected to immunoprecipitation using 2 µg Anti-V5. Immunocomplexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. Western blotting was performed with 0.2 µg/mL Anti-V5. MW shown in kDa. No loading control is presented as matched protein samples, (total protein assayed by Lowry Assay) were resolved in each lane.

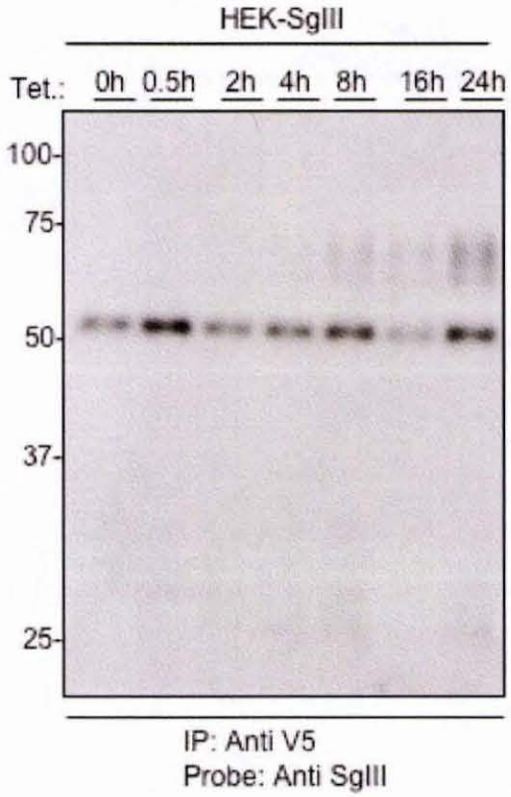


Figure 9c. Kinetics of SgIII over-expression using Anti-SgIII.
The membrane used for western blotting in the kinetic experiment (Figure 9b) with Anti-V5 was stripped and re-probed with Anti-SgIII to verify that the Anti-V5 reactive protein shown in Figure 9b was indeed SgIII. The membrane was probed with 0.26 μg/mL Anti-SgIII. MW shown in kDa.

5.2.4. Production of stable cell lines with over-expression system in PC12 neuroendocrine cells

The construct was next electroporated into neuroendocrine PC12 (Figure 10) cells for the production of an over-expression system. These cells were selected because they represent a different type of secretory system to mast cells. These cells are the cell lines in which Granins have previously been shown to be important (24, 25, 29, 34, 40). Clonal cell lines with over-expression of SgIII were selected by serial dilution in zeocin-containing media, and screened for expression of V5-SgIII by immunoprecipitation and Western blot. Weak production of V5-SgIII protein was observed in this system, despite

very strong expression of the endogenous SgIII observed in Anti-SgIII Western Blot (data not shown).

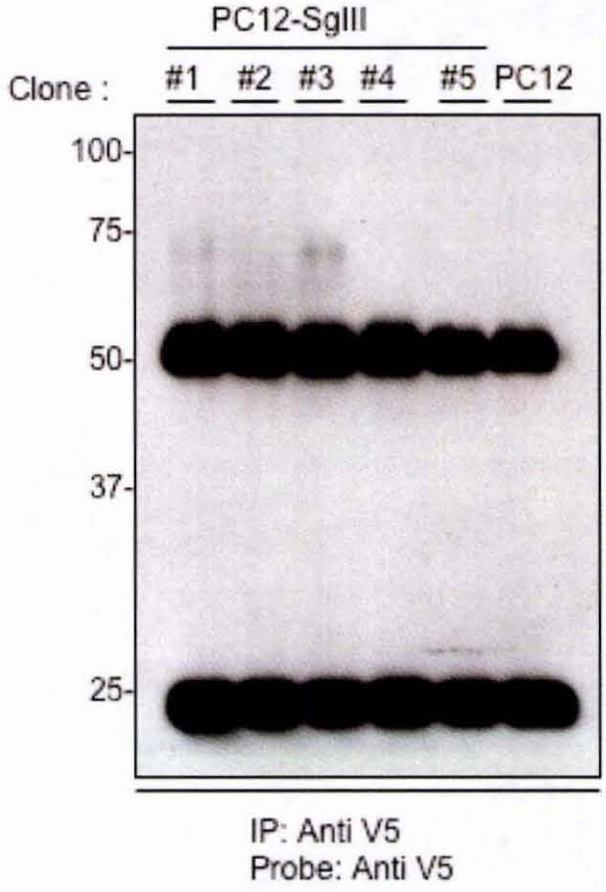


Figure 10. Stable cell lines of PC12 with over-expression of SgIII.
PC12 cells were transfected with the pcDNA4TO-V5-SgIII construct using electroporation, and clonal cell lines were derived by limiting dilution and selected in zeocin media within 14 days of transfection. Cell lysates from control cells (PC12) and transfectants (#1-#5) were prepared and immunoprecipitated using 2 µg Anti-V5. Immunocomplexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. Western blotting was performed with 0.2 µg/mL Anti-V5. MW shown in kDa.

5.2.5. Post-translational modification of V5-SgIII protein.

Biosynthesis of proteins begins in the nucleus with transcription, followed by translation and translocation into the endoplasmic reticulum through the cytoplasm. The proteins can contain sequences or motifs due to which the proteins undergo post-translational modifications as the protein progresses through the endoplasmic reticulum into the

Golgi. This modification results in the attachment of the protein to other biochemical groups such as carbohydrates, lipids, phosphates, and sulfates.

According to our literature survey, Granins are highly post-translationally modified. The common post-translational events studied in these proteins are proteolysis, glycosylation, sulfation, and phosphorylation (27, 48). We proposed that V5-SgIII protein is heavily modified because it migrates as multiple bands above its theoretical molecular weight on SDS-PAGE. We therefore decided to examine the source of these modifications that occur in the later phase of protein production.

Proteolysis or directed digestion of the protein by cellular enzymes or proteases was the first post-translational event that was studied. Proteolysis has been suggested as one of the common features among the Granin family members. We were able to exclude that proteolysis is a major post-translational event for SgIII because the over-expression system of SgIII in mast cells showed no evidence of lower molecular weight forms. It could be possible that the over-expressed protein is not behaving like the endogenous protein. But, previous results of Western blots (Page 27) show that even the endogenous SgIII protein is not proteolysed in mast cells.

There are three major post-translational modifications that could account for the mobility pattern of SgIII on SDS-PAGE.

1. Glycosylation
2. Phosphorylation
3. Sulfation

Proteins are glycosylated as a result of addition of any sugar molecule. Glycosylation is a key modification for Granins, since it is believed to be involved in targeting the proteins to vesicles. We therefore asked if any apparent modifications are due to glycosylation. SgIII contains sites for tyrosine directed kinases. We asked if any of the apparent modifications were due to phosphate group incorporation. It has been stated in literature

that SgIII protein contains a putative tyrosine sulfation site. Sulfation has been particularly interesting because lots of granule components are found to be heavily sulfated and sulfation is shown to play a role in strengthening protein-protein interactions. Secreted proteins and extra-cellular parts of membrane proteins that pass through the Golgi apparatus are known to be sulfated, as are many important components of mast cell secretory vesicle (65).

We hypothesized that the mobility pattern of the full-length SgIII protein can be attributable to the post-translational modifications such as glycosylation, phosphorylation and sulfation. To test this hypothesis we incubated the cells in the absence or presence of the inhibitors of glycosylation, sulfation and phosphorylation. We selected the following inhibitors - tunicamycin, sodium chlorate and genistein. Tunicamycin was used as an inhibitor of glycosylation. It blocks the initial phase of synthesis of *N*-linked oligosaccharide chains resulting in production of glycoproteins with missing or altered chains (58). Sodium chlorate was used to block the tyrosine derived sulfation of the protein (60). Genistein inhibits a range of protein tyrosine kinases and thus down-regulates tyrosine derived phosphorylation events (59). The data suggests that though the protein is not being proteolysed, it is being heavily glycosylated (Figure 11a and b). Figure 11a shows the total protein expression (IGEPAL lysates) whereas Figure 11b shows the immunoprecipitated protein. The cells were also incubated with inhibitors to study the kinetics of the modifications (data not shown). Our data suggested that the full-length protein of V5-SgIII was heavily glycosylated at the 16 h time point after the incubation of the cells with inhibitors.

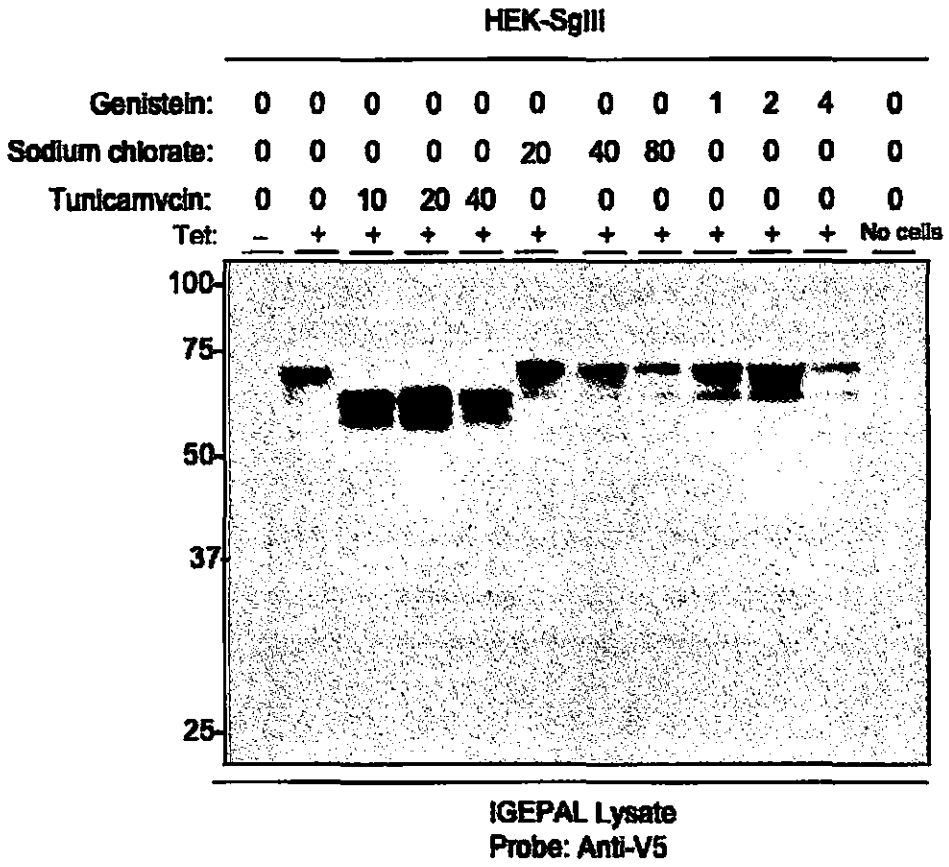


Figure 11a. Inhibition of post-translational modifications of the V5-SgIII protein analyzed by SDS-PAGE of cellular lysates.

HEK-SgIII cells were induced in presence or absence of tetracycline (Tet) and incubated in absence or presence of inhibitors such as Tunicamycin (10, 20, 40 μg/mL), Sodium Chlorate (20, 40, 80 μg/mL) and Genistein (1, 2, 4μg/mL) overnight. The cells were then harvested and IGEPAL lysates were prepared. The total protein was resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. Western blotting was performed with 0.2 μg/mL Anti-V5. MW shown in kDa. Left lane is untransfected cells.

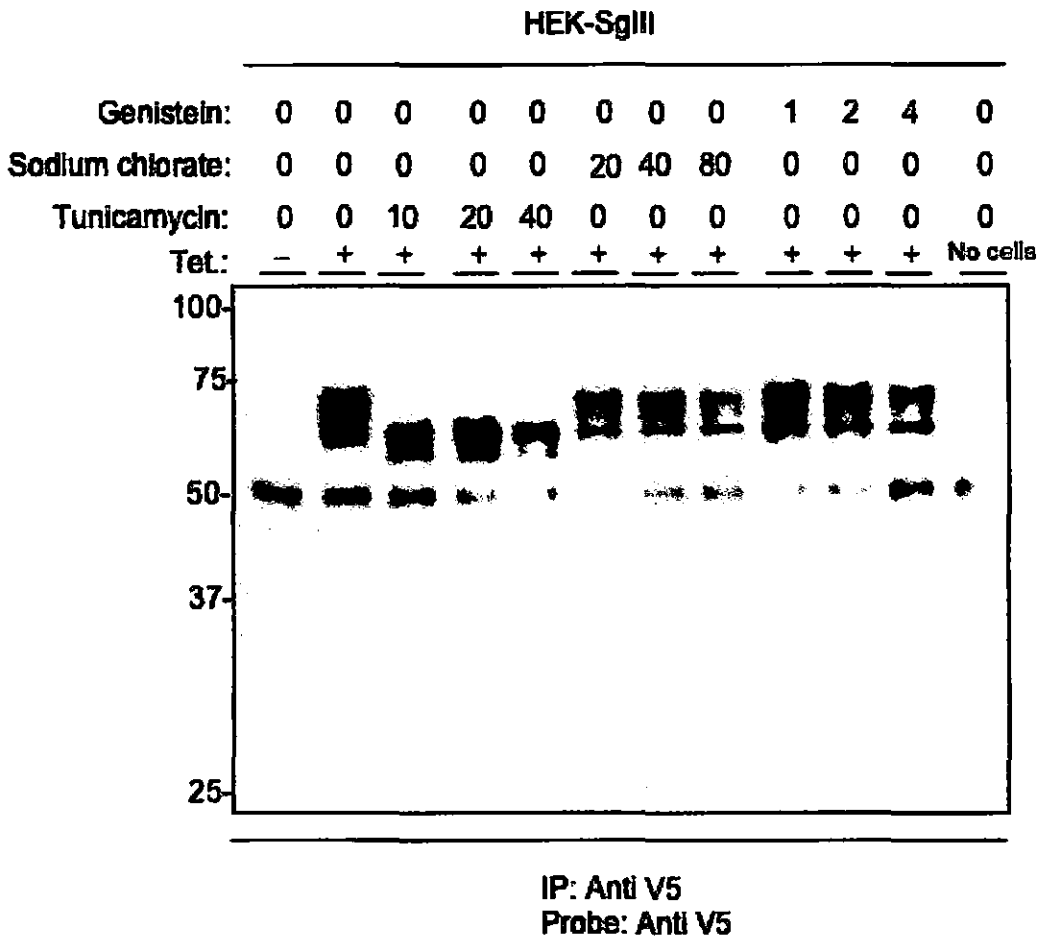


Figure 11b. Post-translational modifications of immunoprecipitated V5-SgIII protein.
HEK-SgIII cells were induced in presence or absence of tetracycline and incubated in absence or presence of inhibitors such as Tunicamycin (10, 20, 40 μg/mL), Sodium Chlorate (20, 40, 80 μg/mL) and Genistein (1, 2, 4μg/mL) overnight. The cells were then harvested and immunoprecipitated using 2μg/mL Anti-V5. The immuno-complexes were resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein. Western blotting was performed with 0.2μg/mL Anti-V5. MW shown in kDa. 50kDa band corresponds to antibody heavy chain. Left lane contains untransfected cells.

5.2.6. Calculated change in molecular weight due to de-glycosylation.

Western blot analysis gave us evidence that the V5-SgIII protein was heavily glycosylated. We therefore wanted to quantify the change caused due to the presence of inhibitors of glycosylation. We also quantified the changes brought about by the presence of other inhibitors suggesting that there were no other post-translational modifications in V5-SgIII protein. To calculate the R_f values we used the raw data from

Figure 11b. R_f value was calculated by dividing the migration distance of the protein with the total distance covered on SDS-PAGE. First a curve using the R_f values of the protein ladder was made. Plotting the calculated R_f values of the protein on this curve enabled us to calculate the shift in molecular mass after treatments with inhibitors of post-translational modifications (Figure 12).

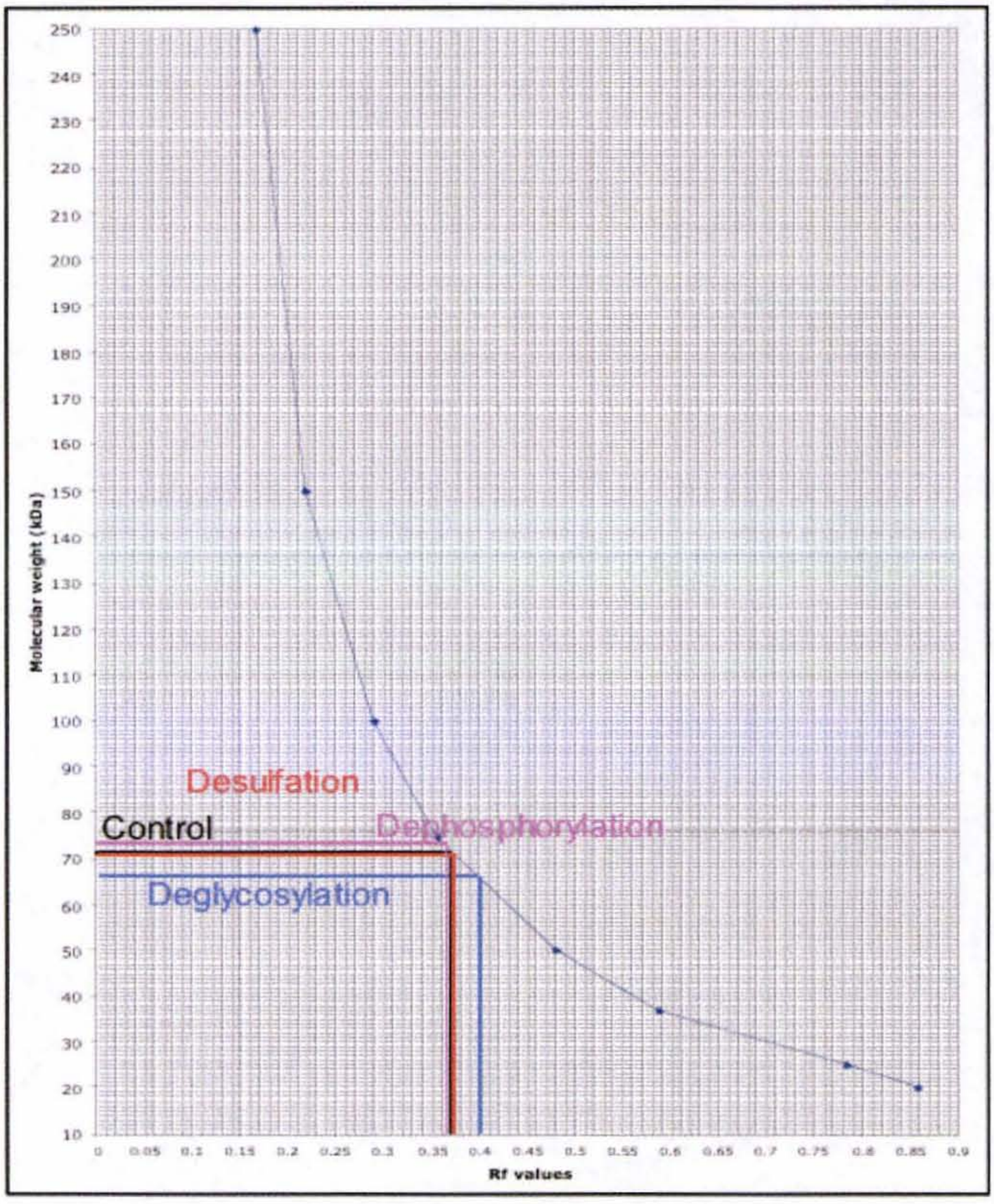


Figure 12. Rf analysis curve.

This curve was made to calculate the shift in molecular mass of SgIII-V5 after treatment with inhibitors of glycosylation, sulfation and phosphorylation. Tunicamycin treatment resulted in apparent decrease of ~7 kDa.

5.3. Discussion.

In this chapter we aimed to develop systems that would constitutively or upon induction, over-express SgIII protein. Based on our results in the previous chapter we showed that SgIII mRNA and protein showed a distinctive expression profile that may reflect it being a component of secretory vesicles. We also showed that the endogenous SgIII protein is expressed in mast cells as well as endothelial cells. We hence hypothesized that SgIII could be a component of the mast cell secretory machinery. Following the above stated hypothesis we proceeded to develop an over-expression system to study the protein in detail.

We successfully produced V5-SgIII over-expression systems for gain of function experiments in three distinct cell backgrounds – RBL2H3 (constitutive over-expression system), HEK293 (inducible system) and PC12 (constitutive system). The PC12 cells were the weakest system among the three systems. Studies on kinetics of protein expression in HEK cells revealed that the protein is expressed after 2 h of induction with tetracycline and that the amount of protein produced at >16 h of induction remained constant. Probing with Anti-SgIII, which is directed against SgIII protein confirmed that the protein being expressed was indeed V5-SgIII protein.

We discovered that there was no proteolysis of the protein into lower molecular weight forms. On protein sequence analysis we found that SgIII protein sequence has 3 sites for *N*-linked glycosylation (68-71, 346-349 and 350-353). Treatment with tunicamycin, an inhibitor of glycosylation decreased the molecular mass by approximate 7000 Da as calculated by the R_f analysis (Figure 12) giving an evidence of V5-SgIII being glycosylated. There was no evidence of phosphorylation of SgIII by tyrosine specific protein kinases or for significant protein tyrosine derived sulfation.

Though it has been reported that SgIII is tyrosine sulfated, we could not find a significant evidence of SgIII being sulfated. Sodium chlorate, the common inhibitor that

was used to inhibit protein tyrosine sulfation, perhaps did not work. We searched for other tyrosine sulfotransferase inhibitors (61). Bertozzi *et al.* generated tyrosylprotein sulfotransferase (TPST) inhibitors by combinatorial target-guided ligand assembly. TPSTs catalyze the sulfation of tyrosine residues within secreted and membrane bound proteins. On contacting Dr. Bertozzi, we were told that these novel inhibitors were not cell permeant and therefore would not be useful to us as SgIII proteins are organized in granules in cytosol of the cell. Future experiments would need to include controls for the action of sodium chlorate, which is cell permeant, and also mutate potential tyrosine sulfation sites. Future studies on glycosylation of SgIII protein might answer the question whether glycosylation is important for the trafficking and function of the SgIII protein.

Chapter 6. Results III: Analysis of SgIII over-expressing cells.

6.1. Introduction.

In the previous chapter we discussed the development and verification of our tools for over-expression of SgIII protein. We developed RBL2H3, HEK293 and PC12 over-expressing V5-SgIII cells. We investigated the kinetics of SgIII protein expression in the inducible over-expression system. This revealed that protein was expressed after 2 h of tetracycline induction and the expression plateaued after 16 h of induction. We also studied the post-translational events in the over-expression system. The results found excluded involvement of proteolysis, protein tyrosine sulfation and protein kinase phosphorylation. Glycosylation was the only evident modification that was observed.

Our next step was to investigate the features of cells that over-express SgIII protein. We noted from previously published studies in neuroendocrine cells that over-expression of Granins can be associated with expansion of vesicular compartments, probably lysosomes (51, 52). We therefore designed immunofluorescence experiments to ask if this phenotype accompanied SgIII over-expression in mast cells.

6.2. Results.

6.2.1. Immunofluorescence analysis

We performed epi-fluorescence microscopy on RBL mast cells and PC12 cells which were either untransfected or constitutively expressed SgIII. HEK-SgIII with, or without, induction were also stained with fluorophores (Figure 13). We stained the cells with Hoechst 33342 stain, which stains the minor groove of DNA, and Anti-V5 followed by fluorescent secondary antibodies to recognize the SgIII-V5 protein. Interestingly, we observed the appearance of new large vesicles, both constitutive and inducible expression systems (Figure 13).

The kinetics of the protein expression was visualized by inducing HEK-SgIII with tetracycline at different time-points (30 min, 2 h, 6h, 16 h, and 24 h). We observed the initiation of granule formation from 2 h after induction with tetracycline as suggested by Western Blot analysis done earlier to study kinetics of SgIII protein over-expression (Chapter 4). We found that HEK-SgIII in absence of tetracycline induction showed no signs of the presence of granules except the primary antibody background as seen in Figure 14 A. The following panels in Figure 14 B-F show the granule formation upon induction with tetracycline. At 24 h after induction we can see the fully formed granules. Interestingly, even at early time-points large vesicles are formed, suggesting that the large vesicles do not arise from fusion of many small granules.

We had previously observed that treatment with tunicamycin disrupted processing and post-translational modification of SgIII. We asked whether tunicamycin treatment had any effect upon induction of vesicle structures by SgIII over-expression. To answer this question we performed the following experiment. RBL2H3 cells over-expressing SgIII were treated with tunicamycin, sodium chlorate and genistein, the inhibitors of *N*-linked glycosylation, protein tyrosine sulfation and protein kinase phosphorylation respectively (Figure 15). Tunicamycin treated cells were observed to have lost their granular structures. SgIII- transfected cells did not exhibit any changes in their granule structures in the presence of sodium chlorate or genistein (Figure 15). The above results gave us a visual idea of the features of the over-expression system, that SgIII over-expressing cells resulted in the appearance of novel granules and that the granular structures were lost when the cells were treated with inhibitors of glycosylation.

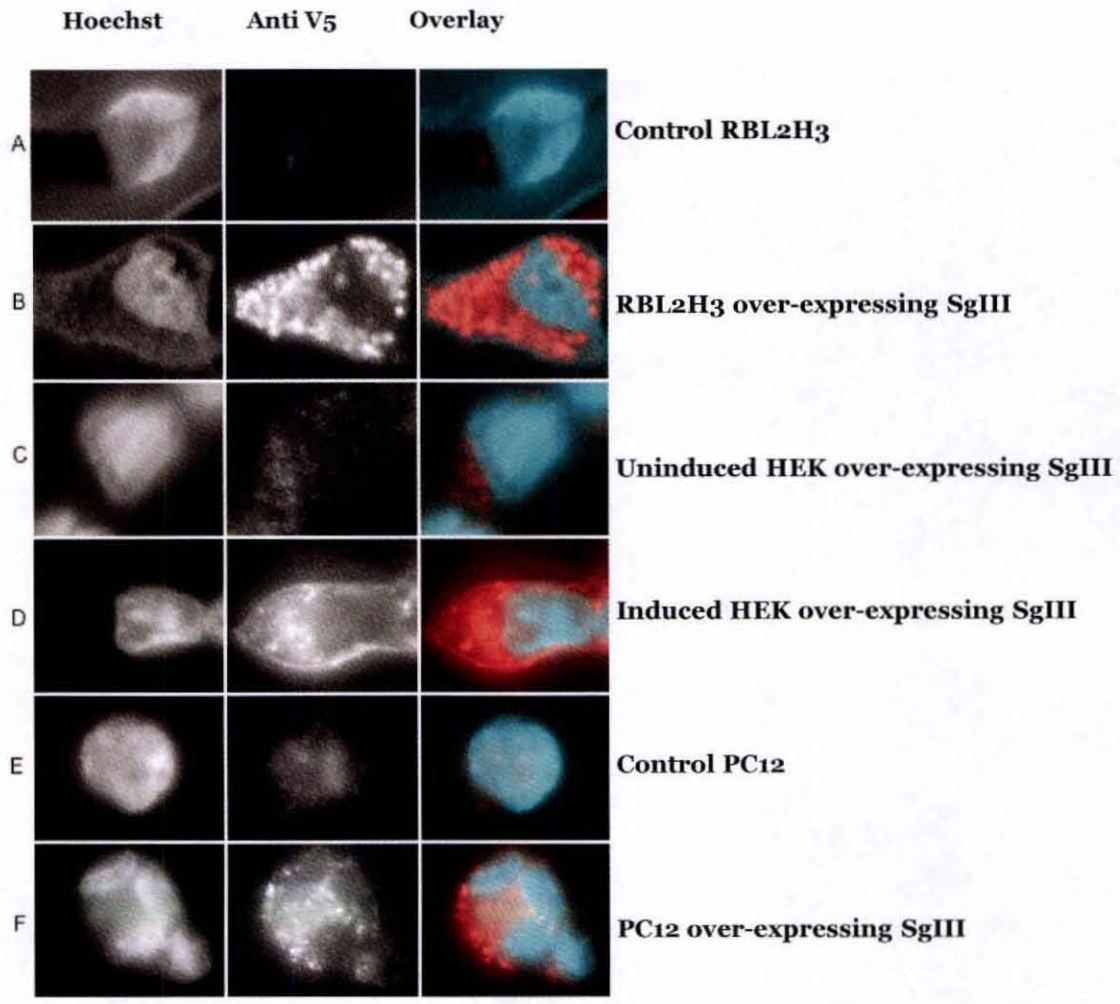


Figure 13. Comparative analysis of SgIII over-expressing cells and non-expressing wild type cells.

A. RBL2H3 cells were grown on cover slips and fixed with methanol. After blocking the cells were stained with Anti-V5 (0.2 μg/mL) and an Alexa 568 nm-conjugated secondary antibody (pseudo-colored red). Nuclei were stained using Hoechst 33342 (100μg/mL, pseudo-colored blue). B. Over-expression of V5-SgIII in RBL cells. C, HEK-SgIII cells without induction and D with tetracycline induction. E Represents PC12 cells and F, PC12-SgIII. Cells in panels D were induced with Tetracycline (1μg/mL) overnight. The left panels are the nuclei, middle panels represent the V5 stained vesicles and the right panels are the overlaid images.

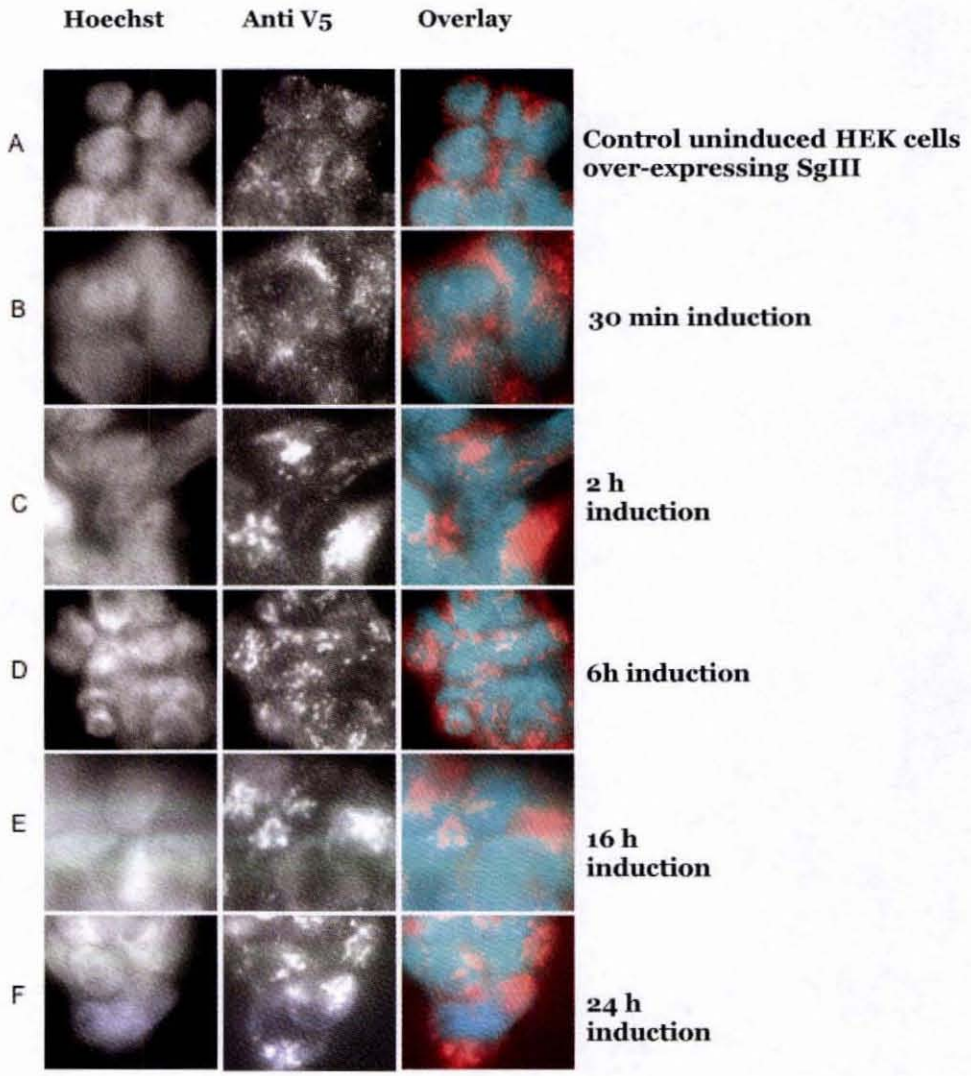


Figure 14. Kinetics of vesicle formation in SgIII over-expressing HEK cells.
A-F, HEK with inducible over-expression of V5-SgIII were grown on glass cover slips and fixed in Methanol. After blocking, V5-tagged proteins were visualized using Anti-V5 (0.2 μg/mL) and an Alexa 568 nm-conjugated secondary antibody (pseudo colored red). Nuclei were stained using Hoechst 33342 (100μg/mL, pseudo-colored blue). A, HEK-SgIII cells were uninduced. B, Cells were induced with Tetracycline for 30 min. C; cells were induced for 2 h, D for 6h, E for 16 h and F for 24 h.

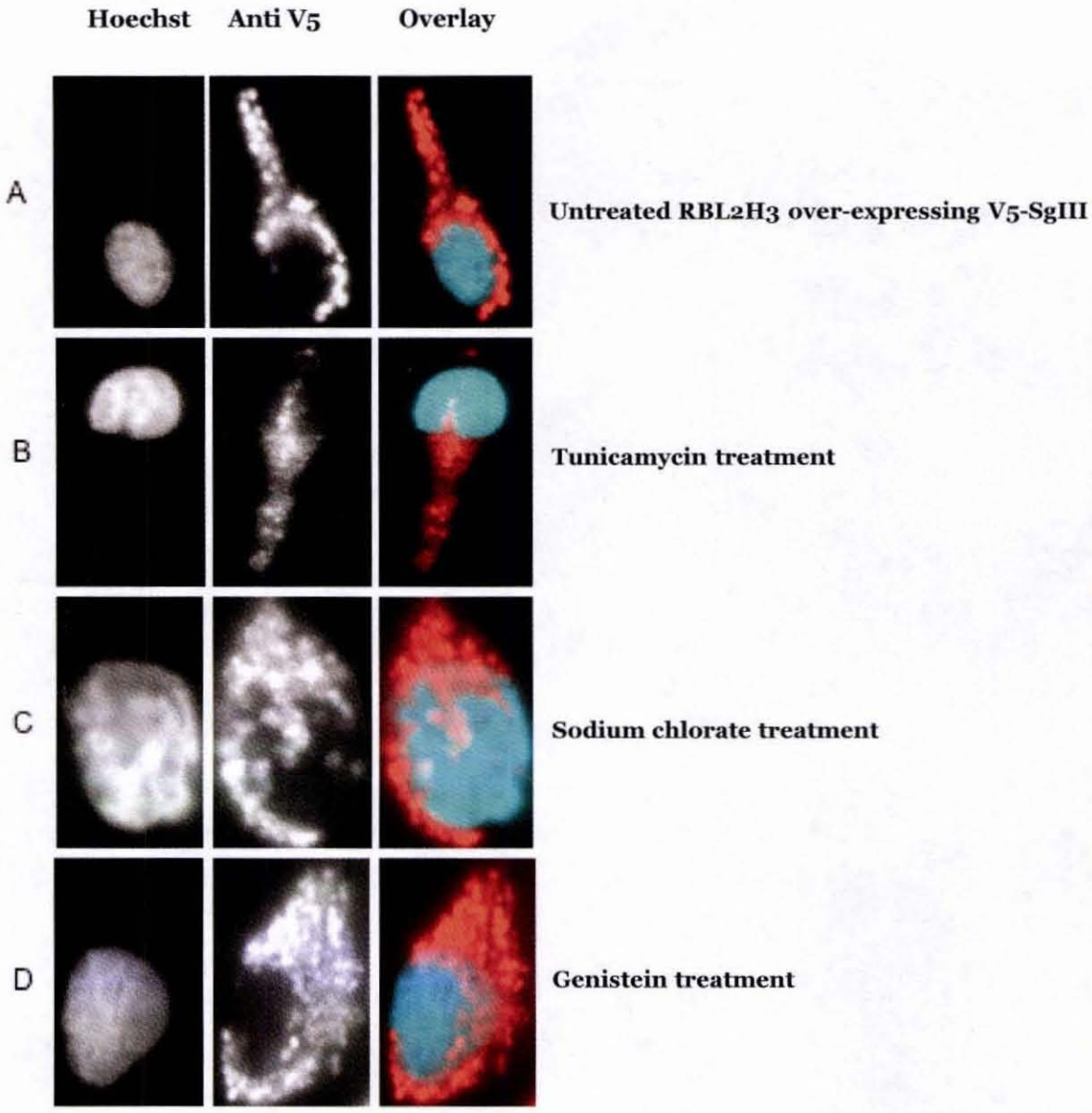


Figure 15. Effects on granule structure in SgIII over-expressing RBL2H3 cells in the presence of inhibitors of post-translational modifications.

A-D, RBL-SgIII cells were grown on glass cover slips and fixed in Methanol. After blocking, V5-tagged proteins were visualized using Anti-V5 (0.2 μg/mL) and an Alexa 568 nm-conjugated secondary antibody (pseudocolored red). Nuclei were stained using Hoechst 33342 (100μg/mL, pseudocolored blue). A; Control RBL-SgIII cells with no treatments. B, Cells were treated with Tunicamycin (10 μg/mL); C, cells were treated with Sodium Chlorate (20 μg/mL); D, cells were treated with Genistein (1μg/mL). All cells were treated with inhibitors 16 h before fixing.

6.2.2. V5-SgIII induced vesicles are depleted following exposure to mast cell activating stimuli.

V5-SgIII expressing RBL cells were shown to give rise to novel granules which seemed to lose their granular structures when treated with inhibitors of glycosylation. The Western Blot analysis of SgIII in mast cells following pharmacological stimulations showed the disappearance and consequently appearance of SgIII after 60 min and 4 h of stimulation respectively. With this idea in mind that SgIII protein levels were depleted following stimulation of mast cells to induce degranulation and were later restored, we designed another immuno-florescence experiment where degranulation in RBL cell over-expressing SgIII was visually studied by stimulating the cells at specific time points with DNP conjugated to BSA thereby cross-linking DNP-IgE bound to FcεRI and stimulating cellular responses (Figure 16). In addition, cells were stimulated with PMA and ionomycin which potently mimic the signaling events that follow FcεRI cross-linking (Figure 16). Our data suggest that the initiation of disappearance of granule structure starts after 20 min of stimulation. At 2 h after stimulation the granule structures were less abundant in stimulated cells, compared with unstimulated cells. This data is again consistent with the mast cell activation kinetics (Figure 3a). The results suggest that the new vesicles generated by over-expressing SgIII in mast cells actually behave like mast cell granules. Upon activation, the granules are apparently exocytosed or their structure is highly disrupted.

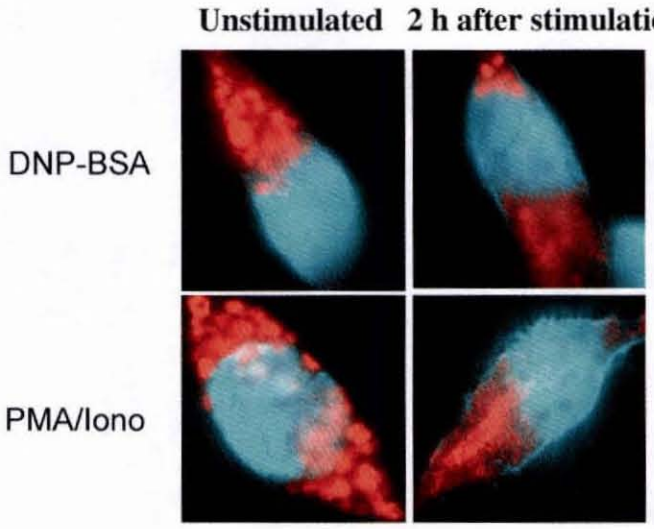


Figure 16. Effects of mast cell activators in granule structure in SgIII over-expressing RBL2H3 cells.

B-E, RBL-SgIII cells grown on glass cover slips were stimulated at 5 min, 20 min, 1h and 2 h with DNP-BSA and PMA/ionomycin. The cells were then fixed in Methanol. After blocking, V5-tagged proteins were visualized using Anti-V5 (0.2 μg/mL) and an Alexa 568 nm-conjugated secondary antibody (pseudo-colored red). Nuclei were stained using Hoechst 33342 (100μg/mL, pseudo-colored blue). A. Control unstimulated RBL cells over-expressing SgIII. B, Cells were fixed after 5 min stimulation; C, cells were fixed after 20 min stimulations; D, cells were fixed after 1h of stimulations and E after 2 h of stimulations. 250ng/mL of DNP-BSA was used and 500nM of PMA and 500nM of Ionomycin were used to stimulate the cells

6.2.3. Expression of lysosomal marker protein in control and SgIII over-expressing cells.

Beige mice have been known to have abnormal mast cell granules. Ultra-structural analyses of secretory cells of beige mice have demonstrated the presence of large lysosome-like vesicles and the enhanced granule content has been attributed to its origin from lysosomes (53, 54, 55). A parallel has been drawn in the literature between the appearance of large granules in beige mice and the appearance of large granules in Granin over-expressing cells. Since the beige phenotype represents lysosomal expansion, it has been proposed that Granin over-expression causes enhanced lysosome biogenesis. The discovery of giant secretory vesicles within secretory cells of beige mouse prompted

us to test the idea in SgIII over-expressing cells if their vesicular structures have lysosomal characteristics.

First we did immunofluorescence to stain vesicles with Anti LAMP2 (Lysosome marker). We saw no co-localization of the Anti-V5 staining and Anti-LAMP2 staining (data not shown), suggesting that these vesicles are not lysosomes *per se*. We set up an experiment with SgIII over-expressing cells and wild type cells, prepared IGEPAL lysates and probed the membranes with Anti LAMP2 which is a commonly used lysosomal marker (Figure 17). Jones *et al.* used this marker to study the presence of lysosome-associated granules in Chediak Higashi lymphoblastoid cell lines (66). Our Western Blot analysis gave evidence of the presence lysosomal marker in slightly greater amounts in the SgIII over-expression system in comparison to the control mast cells. We could not see any striking difference between the control cells and cells over-expressing SgIII in terms of lysosomal marker, suggesting that the lysosome expansion possibility might not be true in the cells over-expressing SgIII.

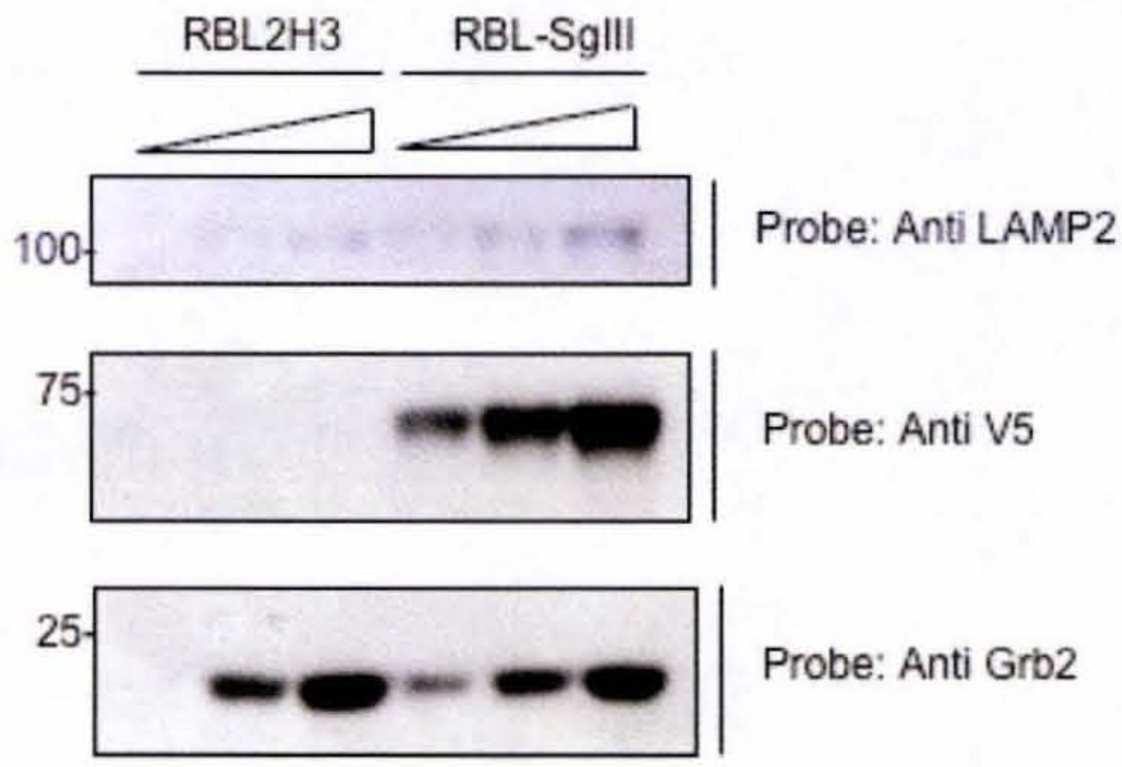


Figure 17. Western blot analysis of Lysosomal marker protein in RBL-SgIII over-expressing cells.

RBL2H3 mast cells and RBL-SgIII cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE in increasing concentrations of the proteins. After electro-transfer and blocking, Western blotting was performed with 10 μg/mL Anti-LAMP2, 0.2 μg/mL Anti-V5 and 0.5 μg/mL Anti-Grb2. MW shown in kDa.

6.3. Discussion.

In this chapter we investigated some prominent features in SgIII over-expressing cells. We discovered the appearance of new vesicles in SgIII over-expressing cells. This feature was common to all the three SgIII over-expressing stable cell lines. The formation of these new vesicles could be traced after 2 h of induction in the inducible over-expression system. The formation of these new vesicles can be attributed to two possibilities. First, either it is the fusion of many small vesicles resulting in the formation of these new large granules. The second possibility is that these large granules are formed right from the beginning of the protein expression. Our kinetic experiments suggest the latter, since we see large vesicles at the earliest time points.

Earlier results on possible post-translational modifications suggested that SgIII protein showed signs of glycosylation. Immunofluorescence experiments were repeated to visualize the effects on granule structures after treatment with inhibitors of post-translational modifications. The new vesicles seem to lose their granular structure when they were treated with inhibitors of *N*-linked glycosylation. There was no notable effect of any other post-translational events studied.

Another experiment was designed to study the exposure of V5-SgIII induced vesicles to mast cell activating stimuli as DNP-BSA, PMA and ionomycin. We noticed that the new vesicles showed depletion in their granule contents resulting in the disruption of the granular structures showing signs of degranulation. There could be two possibilities for these results. First possibility could be that SgIII is being degraded after stimulation with mast cell activators. Yet, there is another possibility that SgIII is being secreted in a manner similar to mast cell proteases. This possibility can be verified by designing an experiment that could detect the secreted SgIII in the extra-cellular media (see Chapter 6).

Another question that arose was whether these granules were originating from lysosomes or had any similarity to other Granin-induced vesicular structures that are thought to be modified lysosomes. Lysosome associated membrane protein (LAMP) expression was verified in IGEPAL lysates of SgIII over-expressing cells but only a slight increase in LAMP levels was observed with the SgIII-V_5 over-expression. Immunofluorescence of the over-expressing cells with lysosome marker showed no co-localization with the novel granules formed (data not shown). Both Western blots and immunofluorescence data were not conclusive of the idea of the granules being formed from lysosomes. There is yet another possibility that the new large granules formed that had lysosomal characteristics were actually formed as a result of fusion of one or more cytoplasmic vesicles. Kinetic experiments in SgIII over-expressing HEK cells after induction suggested that the large granules are formed right from the start of induction and probably do not result from fusion of the small vesicles.

Our next goal is to ask whether vesicles formed in RBL cells over-expressing SgIII protein are perhaps actual secretory vesicles. For this we designed secretory assays, which would give us an idea of the secretory properties of the novel granules formed in mast cells, if SgIII could package the mast cell secretory marker and if it could, then is there an enhanced secretion of this marker when cells are activated? These experiments would also answer the question whether the protein is being secreted into the extra-cellular media or whether it is being degraded after FcεRI stimulation.

Chapter 7. Results IV: Analysis of SgIII-induced vesicles in mast cells over-expressing SgIII.

7.1. Introduction.

In the last chapter we presented data showing that over-expression of SgIII in mast cells leads to the formation of large vesicular structures. We also observed that these vesicular structures were lost when the cells were treated with inhibitors of glycosylation. The vesicular structures were apparently depleted in SgIII over-expressing mast cells when they were exposed to mast cell activators. This chapter investigates whether these SgIII-induced vesicles can package pro-inflammatory mediators such as serotonin. We asked whether the SgIII-induced vesicles contain the pro-inflammatory mediator serotonin. This question was addressed using immunocytochemistry and functional assays. We also asked if SgIII protein was secreted into the external media, as would be expected if SgIII protein is contained within mast cell vesicles.

7.2. Results.

7.2.1. Counterstaining of SgIII-induced mast cell vesicles with Anti-serotonin.

Mast cells store serotonin within their large secretory granules (62). Serotonin is one of the most important vasoactive mediators in an inflammatory site. Since serotonin is a marker for secretory granules, we asked if SgIII induced vesicles contained serotonin. We therefore performed immunofluorescence of SgIII over-expressing RBL2H3 mast cells and control mast cells to look for any packaging of inflammatory mediators in the vesicles of the cells. RBL2H3 control cells and SgIII over-expressing RBL2H3 cells were seeded on cover-slips. The cells were fixed and blocked, followed with Hoechst 33342 staining and 1h incubation with primary antibodies. We used Anti-Serotonin to detect

the serotonin and Anti-V5 to detect the presence of V5 tagged protein in cells. This was followed by the staining the cells with fluorescent secondary antibodies for detection under the microscope. Interestingly, RBL2H3 mast cells over-expressing SgIII, displayed a vesicular population containing high levels of V5-SgIII. We noted that some of these vesicles contained significant serotonin, which co-localized with V5-SgIII, indicating that they may be *bona fide* granules capable of packaging inflammatory mediators (Figure 18).

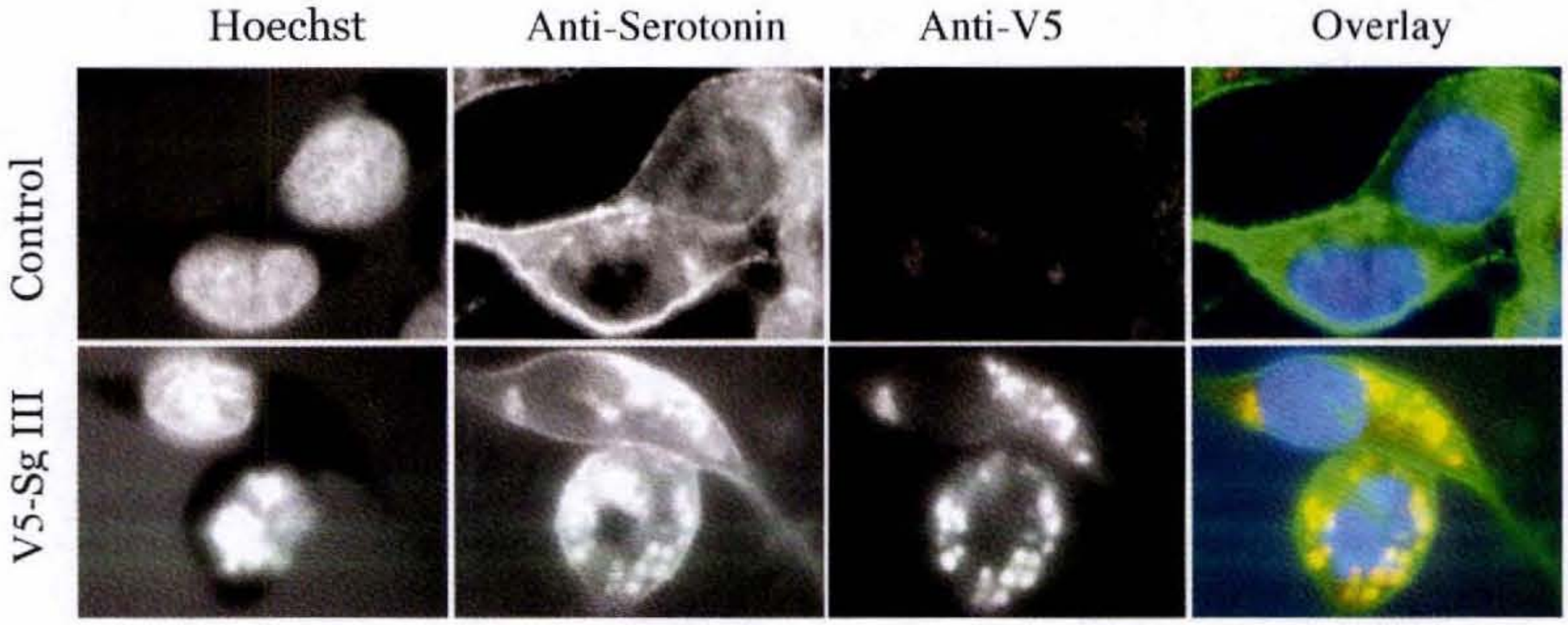


Figure 18. SgIII over-expression results in vesicle formation in mast cells.
Control RBL2H3 (upper panel) and RBL2H3 with constitutive over-expression of V5-SgIII (lower panel) were grown on glass cover slips and fixed in Methanol. After blocking, V5-tagged proteins were visualized using Anti-V5 (0.2 μg/mL) and an Alexa 568 nm-conjugated secondary antibody. Serotonin was labeled using Anti-serotonin (84 μg/mL) and an Alex 488 nm-conjugated secondary antibody. Nuclei were stained using Hoechst 33342 (100 μg/mL). Right panel shows the merged image with Anti-V5 (pseudo-colored red), Anti-Serotonin (pseudo-colored green) and Hoechst 33342 (pseudo-colored blue).

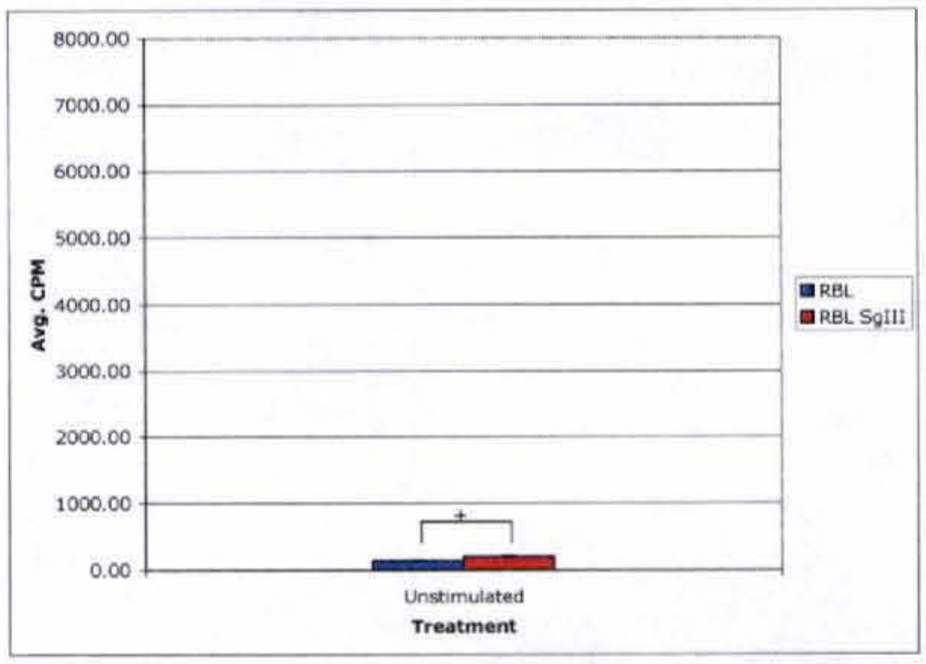
7.2.2. Analysis of secretory responses in RBL2H3 and SgIII over-expressing RBL2H3 cells.

With the idea that V5-SgIII over-expressing granules could package inflammatory mediators such as serotonin, we hypothesized that these V5-SgIII over-expressing mast cells could show an enhanced secretion of the pro-inflammatory mediators. We asked whether the contents of the novel SgIII-containing vesicles could be released in response

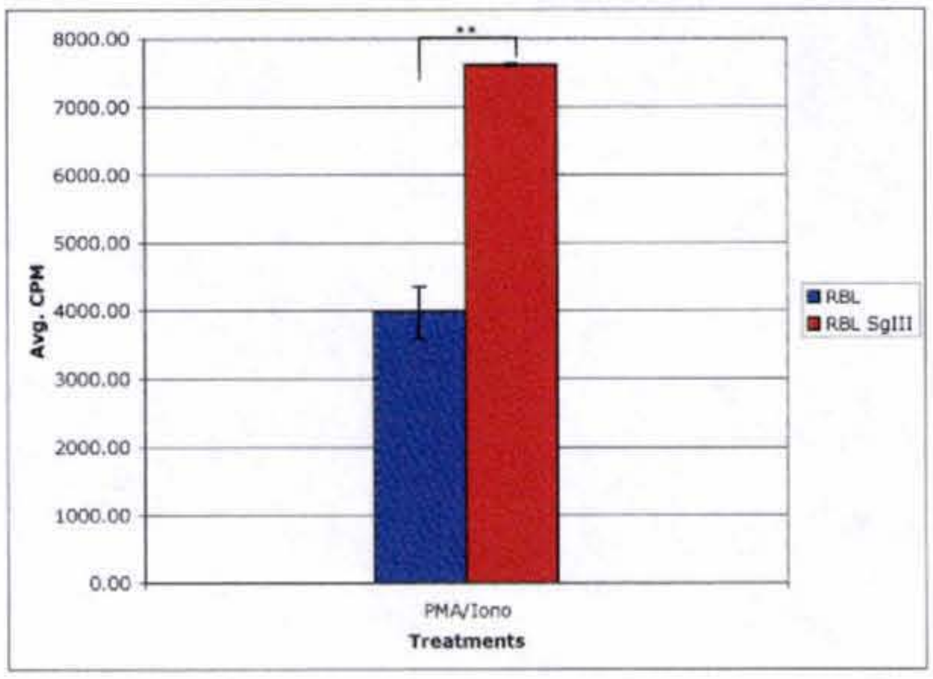
to pro-inflammatory stimuli. RBL2H3 and V5-SgIII over-expressing RBL2H3 cells were used to answer this question. A well-established assay for mast cell secretory responses was employed (64). Here, cells were loaded with ^{3}H-serotonin for 16 h, which is transported into the mast cell granules. After 16 h incubation with ^{3}H-serotonin, mast cells were washed twice and stimulated using antigenic or pharmacological compounds that elicit pro-inflammatory responses. Loading conditions and the washes after the stimulation were designed so that the cells do not have a high basal secretion and that there are no non-vesicular ^{3}H-serotonin leaks from the cytoplasm (64). Degranulation was measured by the appearance of ^{3}H-serotonin in the extra-cellular milieu, which was assayed by liquid scintillation counting.

Figure 19a, shows that there is no significant statistical difference between RBL and SgIII over-expressing RBL cells in terms of ^{3}H-serotonin uptake and basal efflux. We then asked if there was a significant difference when both cell types were exposed to pharmacological activators (PMA/ionomycin) or detergent (Triton). The data in Figure 19b shows that V5-SgIII-expressing mast cells contain enhanced levels of ^{3}H-serotonin, which can be released in response to pharmacological activation (19b). These data suggest that the vesicles arising from V5-SgIII transfection may be functionally equivalent to mast cell granules. Figure 19c too shows a statistically significant enhanced ^{3}H–serotonin content in V5-SgIII over-expressing cells, suggesting a higher level of serotonin incorporation into V5-SgIII over-expressing cells, consistent with the fact that V5-SgIII over-expressing cells contain an expanded granule content.

A



B.



C

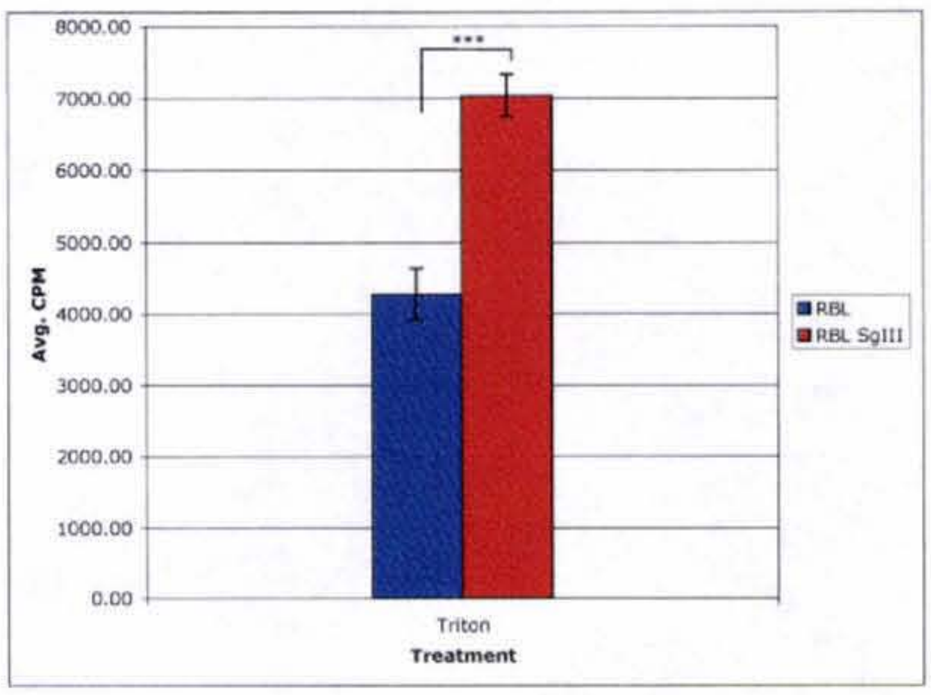


Figure 19. ^{3}H-serotonin release assay.

The Y-axis represents the average scintillation counts per min in 100µl of supernatant from a culture of $1x10^5$ mast cells and SgIII over-expressing cells. **A. Basal secretion of ^{3}H-serotonin from V5-SgIII over-expressing mast cells.** Cells were not stimulated. **B. Enhanced secretory responses in V5-SgIII over-expressing mast cells.** The cells were stimulated with PMA, Phorbol myristate acetate (1µM, 1 hr) and Iono, Ionomycin (10µM, 1h). **C, Enhanced ^{3}H-serotonin content in V5-SgIII over-expressing mast cells** The X-axis represents the presence 10% v/v Triton. Counts from triplicate wells were averaged. Error bars represent standard deviation around the mean. A Student's t-test was done to calculate the *p* values. $+p > 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$

7.2.3. Secreted SgIII can be detected in the extra-cellular milieu of mast cells.

Secretory vesicles follow a regulated secretory pathway, where the secretory vesicles are released from trans-Golgi network and store the newly synthesized proteins, which are then secreted out of the cell by exocytosis. We have shown that SgIII-induced vesicles contain serotonin and that there is an enhanced serotonin secretion in V5-SgIII over-expressing mast cells. We have also shown that SgIII expression follows a specific profile suggesting that it might be secreted during degranulation (Chapter 3). This led us to question if SgIII protein indeed appears in extra-cellular milieu of mast cells. If so, then this would suggest that the data in Figure 5 do not arise from induced degradation of SgIII, but rather from secretion of the protein in secretory granules. With this query in mind, we incubated the RBL and SgIII over-expressing RBL cells (Figure 20). We harvested the un-stimulated supernatants and the cells separately. The supernatant was centrifuged to remove all contaminated cells. IGEPAL lysates of the cell were precipitated on acetone to obtain total proteins. The centrifuged supernatant was precipitated as well on acetone. The proteins were resolved on SDS-PAGE, and probed with the Anti-V5 to detect the V5-reactive proteins. Anti-PDHE1α and Anti-Grb2 were used as controls for cellular contamination in the supernatant. Our data suggests that the V5-SgIII protein is secreted extra-cellularly as a full-length protein. No Anti-Grb2 or Anti-PDHE1α were detected in supernatant implying that there was no cellular contamination and the expression of V5-reactive protein is indeed representing secreted protein in the supernatant. This experiment suggests that SgIII protein was being secreted in the extra-cellular milieu and was not degraded eliminating the possibility of the protein being degraded after exposure to mast cell activators.

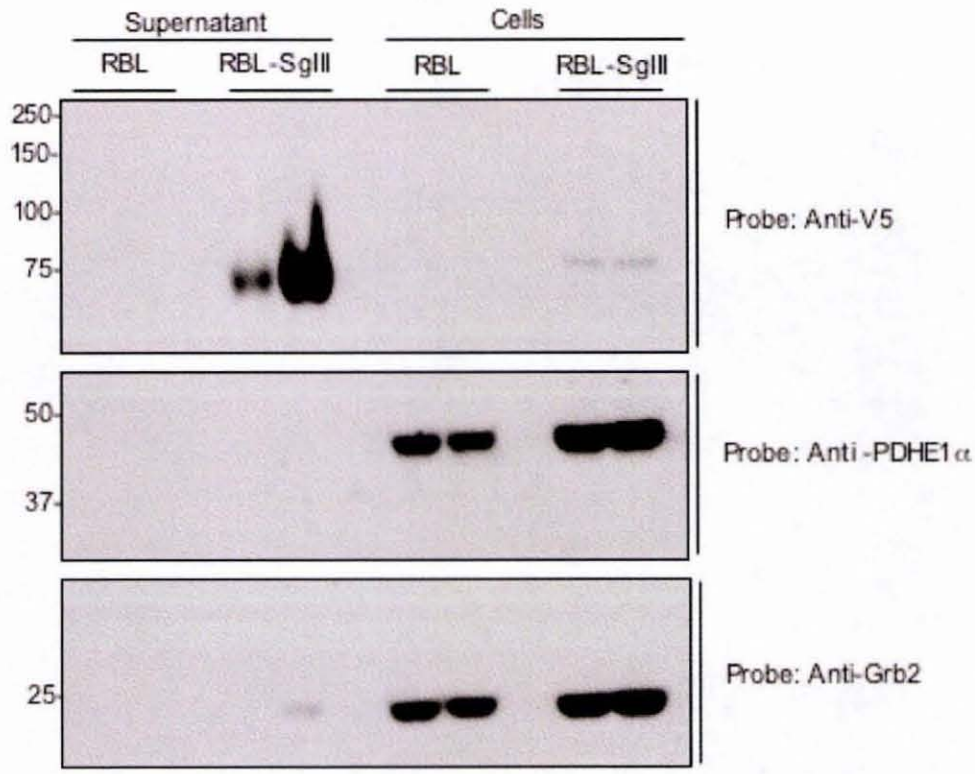


Figure 20. Extra-cellular secretion of SgIII protein in SgIII over-expressing RBL2H3 mast cells.

RBL2H3 and RBL-SgIII cells were plated. The supernatant was collected and centrifuged to discard all the cells. The cells were harvested separately. IGEPAL lysates of both supernatant and cells were made separately and precipitated on acetone for total protein. The total protein obtained was resolved using 10% SDS-PAGE and Western blotted for the presence of V5-reactive protein in the cells and supernatant. Western blotting was performed with 0.2µg/mL Anti-V5, 0.5µg/mL Anti-PDHE1α and 0.5µg/mL Anti-Grb2. MW shown in kDa.

7.3. Discussion.

This chapter focused on the secretory aspect of the SgIII protein. In Chapter 3 we found that SgIII followed a specific bell-shaped expression profile similar to mast cell proteases. Exposure to mast cell activators resulted in the disappearance and consequently re-appearance of SgIII protein expression in mast cells. This observation gave us two possibilities to explain this expression pattern. The first possibility is that the SgIII protein was degraded after exposure to the mast cell activators and, the second

possibility is that the protein was being secreted extra-cellularly into the media and reappearing after 4 h of stimulation. This prompted us to study the secretion of SgIII and verify if the novel granules formed by over-expressing SgIII in mast cells were indeed *bona fide* secretory granules like mast cell granules. The large vesicles produced in SgIII over-expressing mast cells were found to package serotonin, which is one of the most important inflammatory mediators and a secretory marker in mast cells. This feature of the SgIII induced vesicles was similar to mast cell granules.

This observation of SgIII induced vesicles being capable to package serotonin prompted us to ask yet another question, if these granules that could package serotonin, showed an enhanced serotonin release as well, after being stimulated by mast cell activators. If these granules are true secretory granules, we would expect them to exhibit an enhanced secretion. Experiments done to measure degranulation, by secretion assays on SgIII over-expressing mast cells, and control mast cells, showed an enhanced serotonin secretion in response to activators (like PMA and Ionomycin) or when lysed with triton (non ionic detergent that disrupts the integrity of the cell membrane and lyses them), in comparison to the normal mast cells. This enhanced secretion was found to be significant according to the statistical analysis. The enhanced serotonin secretion can also be attributed to the large size of the granules that could package the pro-inflammatory mediator.

Our Western blot analysis of the total protein of cell lysates and supernatant suggested that SgIII protein is secreted extra-cellularly into the culture media. This suggests that vesicles formed in SgIII over-expressing cells might be secretory granules that could store inflammatory mediators. This result decreased the likelihood that the SgIII protein is being degraded after the cells are stimulated with mast cell activators. The reappearance of the protein after 4 h gives evidence that the bell shaped mRNA and expression profile was indeed reflecting replenishment of secreted protein stocks. It

confirmed that the new granules produced in the over-expressing SgIII mast cells were indeed secretory vesicles like the mast cell secretory vesicles and hence our hypothesis that SgIII protein is a component of mast cells secretory machinery may be true.

This chapter suggested that SgIII induced vesicles were indeed secretory in nature and had the capability to package inflammatory mediators. The SgIII protein was secreted extra-cellularly into the media after being exposed to mast cell activators, and its levels are replenished over a time course, similar to that seen for mast cell proteases. Hence our next goal is to study the changes brought about in mast cells when the mRNA responsible for producing SgIII was silenced. This would give us an idea if SgIII protein is indeed a necessary component of the mast cell secretory machinery.

Chapter 8. Results V: Suppression of SgIII expression in mast cells.

8.1. Introduction.

In the previous chapters we discovered that over-expressing V5-SgIII protein in RBL2H3 mast cells led to the formation of new vesicles. These novel vesicles showed properties similar to mast cell granules after stimulation with mast cell activators. Interestingly, these vesicles have shown the capability to package mast cell inflammatory mediators. These vesicles showed an enhanced secretion of the inflammatory mediators giving an idea that these vesicles might really be *bona fide* granules containing the mast cell mediators. We also showed that the SgIII protein was secreted extra-cellularly in the culture media explaining the disappearance of the protein when the cells are stimulated with mast cell activators and undergoing degranulation.

Here, we felt that we established that the protein is a component of secretory machinery in mast cells. So, then we asked whether the protein was necessary for the secretory capability of mast cells. For this we planned some loss-of-function experiments for which we adopted RNA interference techniques. These experiments were aimed to answer the following questions: (1) will loss of SgIII protein cause a defect in secretory functions in mast cells? ; and (2) is SgIII a necessary component of the mast cell secretory machinery or is it just a bystander that gets simply packaged into secretory granules?

RNA interference techniques allow us to down regulate the expression of a specific gene in living cells by introducing a double stranded RNA that is complementary to the target mRNA of interest. Our literature survey suggests that siRNA can be a potent mediator of the RNA interference effects in mammalian cells (63). We chose the siRNA technique as it could knock down the mRNA enabling us to study the properties of a SgIII deficient system. We used a lipid mediated Silencer siRNA Transfection II Kit (Ambion, Texas) that is known to be effective for most of the cell types. This kit provides us with

transfection reagents that are known to have minimal cytotoxic effects. This kit also includes a negative siRNA control, which is a scrambled sequence that bears no homology to the human, mouse or rat genomes. We used a newly developed transfection method called neofection, in which the cells are transfected as they adhere after trypsinization or passages rather than after cell adherence in the traditional transfection technique. This method bypasses several steps of the traditional transfection method, making it faster and easier. We first tested the efficiency of the siRNA in entering the cells, thereby optimizing the conditions, for which we used fluorescently labeled siRNA. Once we had confirmed that cells were absorbing siRNA, we used the human and rat SgIII siRNA to attempt the specific suppression of SgIII expression. The eventual goal of these experiments would be to perform secretion assays in cells with suppressed expression of SgIII compared with wild type cells.

8.2. Results.

8.2.1. Transfection of fluorescent siRNA to optimize conditions.

We transfected RBL2H3 cells, HEK 293 cells and PC12 cells with Silencer ™Cy3-Labeled siRNA, which is an annealed and HPLC-purified siRNA. Labeled siRNA allow for direct observation of cellular uptake, distribution and localization of siRNA of interest, most importantly they allow us to monitor transfection efficiency using a fluorescence microscope during the optimization process. In general, siRNA are rapidly taken up by cells (<4 h) and distributed throughout the cell. We counted the red fluorescent (568 nm) cells after 2 h and 24 h of transfection. Our observation was that Cy3 labeled siRNA penetrated almost 30% of PC12 cells at the 2 h time point and over 80% at the 24 h time point. HEK cells were less efficient with around 10% penetration at 2 h time point, where as the penetration increased to 50% at 24 h time point. RBL2H3 cells were found to be

the most difficult cell lines in terms of their transfection efficiency. Unlike the other cell lines, RBL cells showed no signs of penetration of the Cy3 labeled siRNA at 2 h time point, although siRNA penetrated in 30% of the cells at 24 h time point. This suggested that RBL2H3 cells were relatively poor at siRNA uptake.

7.2.2. siRNA-mediated knockdown of SgIII in RBL2H3 mast cells and PC12 cells.

Our goal was to study the importance of SgIII protein as a component of mast cell secretory machinery. We therefore first performed knock-down experiments in RBL2H3 cells despite the possible difficulties in transfection suggested by previous data. We tried different methods of knocking down SgIII in RBL cells trying to optimize the siRNA transfection into the mast cells. We adopted the Silencer siRNA Tansfection II Kit (Ambion, Texas) protocol and started the transfections with different origins of siRNA (Human and Rat) to knock down SgIII expression so as to proceed further with the most efficient siRNA. Unfortunately there was no notable knockdown of SgIII expression with either of the siRNA used (Figure 21). We observed no knockdown of SgIII levels with any concentration (5nM, 10nM, 30nM and 100nM) of siRNA used. At this point we assumed that probably the transfection kit was not suitable for knocking down the specific gene or mast cells needed different transfection conditions. Hence, we tried another method using a different transfection reagent. We noted a published study where the laboratory of Dr. R. L. Stevens had apparently performed a successful siRNA mediated knockdown in RBL2H3 cells using liposome transfection approach (57). The liposome-siRNA complex was formed by using LipofectAMINE™ 2000 (Invitrogen, CA). This complex was added to the adherent RBL2H3 cells unlike in neofection where the siRNA-Transfection complex is added as the cells adhere to the surface. We therefore decided to mimic this approach. Our results still did not show any success in knocking down SgIII

(Figure 22). We next tried the electroporation method to knock down the specific gene for SgIII in RBL2H3 cells, which are easily electroporated. Electroporation is a standard method for cDNA transfection in these cells. We again were unsuccessful in knocking down the gene expression of SgIII in mast cells (Figure 23). At this point we suggested two possibilities for the lack of knockdowns in mast cells. First, either siRNA was not entering RBL2H3 mast cells or, second, that siRNA upon entry into mast cells, failed to suppress SgIII expression.

We could test the latter by asking if any of the SgIII siRNA that we had designed were capable of knocking down the expression of SgIII in another rat cell line where siRNA access is not a significant problem. Fluorescently labeled siRNA experiments suggested that PC12 cells had good uptake of siRNA. We therefore performed lipid-mediated transfections in PC12 cells following the neofection approach. The cells were mixed with the transfecting agent and siRNA (100nM) mixture and plated and incubated for 72 h. We used both, SgIII specific siRNA and also a Silencer Negative Control siRNA. We observed knockdowns at 10nM, 30nM and 100nM, with 100nM being the most specific and effective concentration of siRNA. Interestingly the specific knockdown with 100nM SgIII siRNA concentration showed no gross changes in the negative controls when compared to the untransfected cells (Figure 24). This suggested that the siRNA-transfection using the neofection approach does work at least in PC12 cells, most likely ruling out one of the possibility of lack of suppressed expression of SgIII in RBL2H3 mast cells. Our central problem hence is probably a lack of access to mast cells.

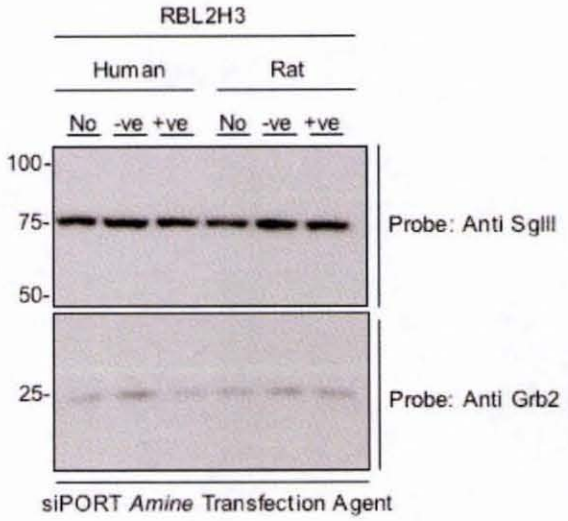


Figure 21. Neofection approach using siPORT Amine Transfection Agent to knockdown SgIII in RBL2H3 mast cells.

RBL2H3 cells were incubated with 30nM SgIII siRNA-siPORT Amine transfection complex for 72 h. Cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 μg/mL Anti-SgIII and 0.5 μg/mL Anti-Grb2. +ve indicates the presence of siRNA specific to SgIII and –ve indicates the non specific control siRNA. No indicates the absence of siRNA. We used siRNA of human and rat origin. MW shown in kDa.

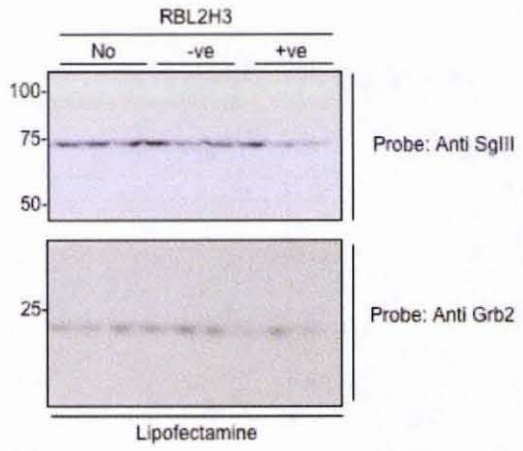


Figure 22. Liposome-mediated Transfection Approach using LipofectAMINE™ 2000 to knockdown SgIII in RBL2H3 mast cells.

RBL2H3 cells were incubated with 40nM SgIII siRNA-lipofectamine reagent complex for 72 h. Cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 μg/mL Anti-SgIII and 0.5 μg/mL Anti-Grb2. +ve indicates the SgIII specific siRNA and –ve indicates the non-specific control siRNA. No indicates the absence of siRNA. MW shown in kDa.

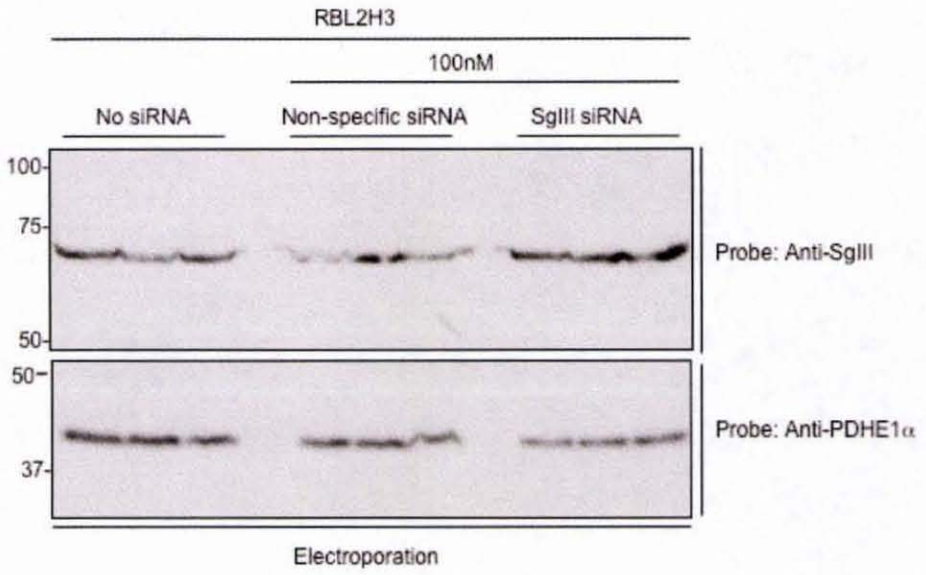


Figure 23. Electroporation approach to knockdown SgIII in RBL2H3 mast cells.
RBL2H3 cells were electroporated and plated with SgIII siRNA and incubated for 72 h. Cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 μg/mL Anti-SgIII and 0.5 μg/mL Anti-PDHE1α. SgIII siRNA indicates presence of siRNA specific to SgIII and Non-specific indicates control siRNA. No indicates absence of siRNA. MW shown in kDa.

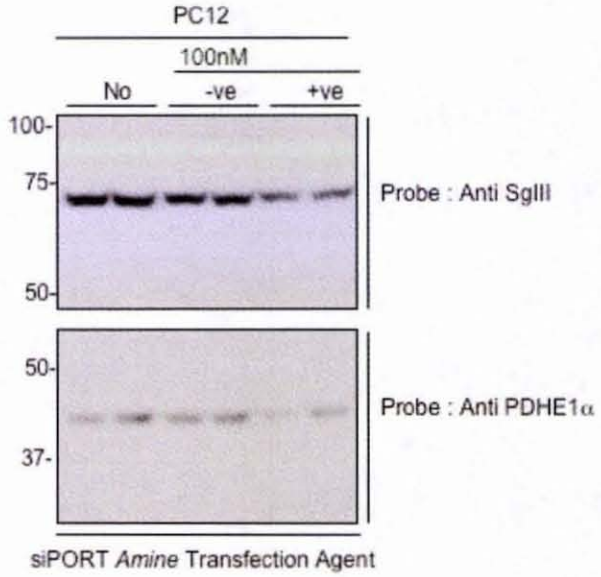


Figure 24. Neofection approach for RNA interference mediated knockdown of SgIII in PC12 cells.
PC12 cells were plated with SgIII siRNA and incubated for 72 h. Cells were harvested and IGEPAL post-nuclear lysates were prepared. Total protein was recovered by acetone precipitation and resolved by 10 % SDS-PAGE. After electro-transfer and blocking, Western blotting was performed with 0.26 μg/mL Anti-SgIII and 0.5 μg/mL Anti-PDHE1α. +ve indicates the SgIII specific siRNA and –ve indicates the non specific control siRNA. No indicates there were no siRNA added. MW shown in kDa.

8.3. Discussion.

We observed in our previous chapter that SgIII containing vesicles in mast cells were indeed secretory in nature and could package inflammatory mediators like serotonin. This chapter discusses about loss of function experiments that were aimed to study the importance and necessity of SgIII protein as a component of the secretory machinery in mast cells. The goals of the experiments performed were to produce SgIII knockdowns. The technique that we adopted was the siRNA technique for SgIII gene silencing which according to our literature search seemed to be very specific in nature. Our literature survey suggested that RBL2H3 cells were difficult cell lines for knockdown experiments. Fluorescently labeled siRNA experiments visually enabled us to analyze siRNA uptake in the three cell lines – RBL2H3, HEK293, and PC12 cells that we used as our background cells for the entire study. Observations showed that RBL cells were very slow and inefficient in the uptake of fluorescently labeled Cy3 siRNA, in comparison to PC12 cells which were most efficient in the uptake among the three cell lines at 2 h and 72 h.

Western blot analysis performed to study the SgIII knockdown in RBL cells suggested that RBL cells either would not take in the siRNA or either SgIII siRNA could not suppress the SgIII gene upon entering the cell. We tried the new neofection method that has been suggested to be the efficient method but without success. We also mimicked the RNA interference experiments using the liposome transfection approach by Stevens *et al.* Though Dr. Stevens' lab was successful in making knockdowns in RBL2H3 by using the Lipofectamine reagent, we were still unsuccessful. We even tried electroporating the SgIII siRNA into the RBL2H3 cells but unfortunately without success. At this point we were confident that one of the two possibilities was true. Either the siRNA could not gain access into the cell or SgIII siRNA could not suppress the SgIII gene in RBL2H3 mast cells. We could test whether there is an access problem for siRNA in RBL2H3 by asking if

any SgIII siRNA were capable of knocking down SgIII expression in any other rat cell line where siRNA access was not a significant problem.

We next tried the PC12 cells, which according to fluorescently labeled siRNA uptake experiments were shown to be the most efficient cells among all three cell lines studied. We again started the knockdown experiments in PC12 cells with the neofection approach using the Transfection kit containing the lipid mediated transfecting reagent. Observations suggested that the knockdown approach worked at all the different concentrations of siRNA - 5nM, 10nM, 30nM and 100nM. The siRNA uptake which was most specific in knocking down the SgIII expression in PC12 cells was observed at 100nM concentration. This meant that we were unable to optimize the uptake conditions in RBL2H3 mast cells. Though we could analyze the sufficiency of the proteins discussed in the past chapters, we were unable to study the knockdown system and hence the complete characterization involving the regulation and function of the protein could not be studied. Interestingly even though we have some knockdown of SgIII expression in the PC12 cells, it is still not specific, so even more optimization is required.

Future experiments on gene silencing in RBL2H3 mast cells needs to be done in order to study the importance of SgIII protein in mast cells. The design of these experiments will need to optimize all conditions for transfection. The amount of transfection reagent is considered as the most important parameter among all other parameters. All other parameters though important can be considered as fine-tuning adjustments. The volume of transfection reagent, starting from the minimum volume, should be optimized such that it forms an optimal liposomal-siRNA complex for the successful transfer into the cells and have minimal cytotoxic effects on the cells. The next important parameter that needs to be optimized is the time for which the cells are exposed to the transfection complex. The amount of siRNA is another important parameter that needs to be optimized.

siRNA experiments in RBL2H3 suggested that somehow the SgIII siRNA could not gain access into the cells and hence optimizing the transfection conditions could be the solution to our problem. However, the fact that even electroporation of siRNA failed in RBL2H3 cells does not give us the cause for optimism. Once the above parameters are optimized, the optimal number of cells transfected also needs to be established, keeping in mind the state of the cells and their reproducing ability. A balance needs to be established between cytotoxicity and activity of transfected RNA. Other alternate approaches that can be considered include the production of stable shRNA cell lines (67) or the use of a traditional antisense approach.

Chapter 9. General Discussion

Mast cells contain granules that package potent inflammatory mediators. These mediators are released into the environment or surrounding tissues upon activation. Kinetic experiments on degranulation and re-granulation of mast cells suggested that the granule contents are depleted in the first 2-30 min after stimulation by mast cell activators, after which the cells enter a refractory phase when the cells are engaged in replenishing its granule contents. The released contents are restored into the granules after 6-9 h of stimulation and the cell then becomes prepared for the next activation cycle.

Granin family members are known to be present in the secretory granules of a wide variety of endocrine and neuro-endocrine cells (23-26). Some members have been shown to be acting as an on/off switch for granule biogenesis (CgA, 29, 30). CgB has been suggested to be involved in secretory granule biogenesis. There has been no published analysis of expression or function of Granins in mast cell system though their involvement in secretory vesicle biogenesis in other secretory cell types has been evident. We hence hypothesized that Granins could be components of the secretory machinery in mast cells.

Transcriptional array analysis showed that transcripts of mast cell proteases showed a specific bell shaped expression after stimulating them with mast cell activators. Interestingly transcripts of only one of the Granin members, SgIII showed this same bell-shaped expression. This observation raised the intriguing question of whether SgIII protein could be a component of the mast cell secretory machinery. Moreover the SgIII protein levels in mast cells showed depletion, after being activated by mast cell activators in the first 60 min -120 min after stimulation. The levels seemed to be restored eventually after 4 h - 24 h after stimulation. Our observations also suggested that SgIII was abundant in each of the mast cells that were studied.

The above results led us to eventually hypothesize that SgIII protein might be a component of the mast cell secretory machinery. In order to study the characteristics of the protein, a construct was developed that upon stable transfection in mast cells and other cells would over-express SgIII. This over-expression of SgIII protein in mast cells would enable us to study the features of the protein when over-produced in the cells.

SgIII cDNA with a V5 epitope on its extreme C-terminus was sub-cloned into pcDNA 4T/O vector. Transient transfection of the construct into HEK cells suggested that the construct was good and that it produced full-length of the protein of the desirable molecular weight. We decided to study the over-expression of the protein in both constitutive expression system as well as an inducible expression system. We developed over-expression systems in RBL2H3 mast cells, HEK293 endothelial cells and PC12 neuroendocrine cells. We were successful in getting several positive clones in all the above cell lines. Over-expression of V5-SgIII protein in inducible T_{REX}HEK cells assisted us in studying the kinetics of the protein expression. Our data suggested that protein was over-expressed after 2 h of induction with tetracycline and reached a plateau at 16 h after induction with tetracycline.

We noticed the migration of the V5-SgIII protein as multiple bands suggesting that the protein was heavily modified. Post-translational events as proteolysis and modifications like glycosylation, tyrosine kinase phosphorylation and tyrosine sulfation were studied. Our results showed no proteolysis of protein supporting the published data of Ottiger *et al* that SgIII was not proteolysed in a manner similar to other Granins. Treatments of the V5-SgIII over-expressing cells with inhibitors of the above listed modifications suggested that the only modification that was notably evident was glycosylation. We used tunicamycin as the inhibitor for *N*-linked glycosylation, which blocks the process in the early stages of this modification. Protein sequence analysis showed that there are three possible sites for *N*-linked glycosylation in human SgIII. Published literature suggests

that SgIII is a tyrosine-sulfated protein like many other proteins found in secretory granules. We used sodium chlorate as an inhibitor for tyrosine Sulfation, mimicking the experiment performed by Mintz *et al* (60). Unfortunately we could not see any changes by inhibiting tyrosine sulfation suggesting that perhaps sodium chlorate failed to work on the SgIII protein. Future works on sulfation needs to have controls for the effect of sodium chlorate. For example we could assay the sulfation status of the sulfated Chromogranin B protein, which is expressed in mast cells (data not shown).

Our immunofluorescence study is performed on SgIII over-expressing cells (RBL2H3, HEK, and PC12) showed the formation of new vesicles in these cells. The kinetic experiment in SgIII over-expressing HEK cells suggested the formation of visible granules as early as 2 h of tetracycline induction. The granules seemed to be large at the earliest time points ruling out one possibility that the new vesicles formed as a result of over-expression of SgIII proteins were a result of fusion of many small vesicles. Treatment with an inhibitor of *N*-linked glycosylation – tunicamycin, showed the loss of the granule structures in mast cells over-expressing SgIII, suggesting that the formation of these granules is dependent more on glycosylations, although we do not yet know if glycosylation of SgIII is required. Though SgIII has been shown to be tyrosine sulfated (48) we have no evidence of this post-translational modification in V5-SgIII over-expressing RBL2H3 mast cells. We also did not see any evidence of tyrosine kinase directed phophorylation in the SgIII over-expressing cells suggesting at this point that *N*-linked glycosylation is the only post-translational modification present on SgIII proteins. Future experiments could map the sites of *N*-linked glycosylation and determine the functional role of the protein or in the trafficking of the protein to vesicles.

Surprisingly we found that the granule structures were less abundant in SgIII over-expressed RBL2H3 cells when they were treated with activators of mast cells suggesting that SgIII protein could be a component of the mast cell secretory machinery, as they

were being secreted into the extra-cellular media in response to activators of mast cells. There is another possibility that the protein was being degraded in the cytosol after stimulation but this possibility was ruled out by the later experiments where we detected the SgIII protein in the supernatant.

Immunofluorescence experiments were performed to test the possibility of the origin of new granules from lysosomes. Our data showed no co-localization of a lysosomal marker protein and the new granules. Western blot analysis of expression of lysosomal marker in SgIII over-expressing RBL2H3 cells suggested slight increase in LAMP2 expression in V5-SgIII over-expressing cells compared with control cells. There seems to be no striking difference between the proteins from control cells and the SgIII over-expressing cells. This suggested that the origin of the new granules formed as a result of SgIII over-expression was not due to any lysosomal expansion.

Electron microscopy revealed a resemblance of mast cell granule to the new granules formed as a result of over-expressing SgIII protein in mast cells. The granules in the RBL-SgIII showed the same dense granular appearance as mast cell granules (data not shown). Noting that the ultra-structure of SgIII-induced and normal mast cell granules were similar we decided to study the secretory aspects of the protein.

We hypothesized that since these granules were very much similar to mast cell granules, then these granules should also package serotonin, like mast cell granules. Immunofluorescence experiments were repeated again, but this time with dual antibodies, one of which was against serotonin, one of the most potent pro-inflammatory mediator and the other to detect the Anti-V5 reactive protein. Interestingly, we found that the new granules formed in the over-expressed cells could package serotonin.

The capability of the new granules formed by the SgIII over-expression in RBL2H3 mast cells raised another question. Could these new granules show an enhanced serotonin release upon being stimulated by mast cell activators (PMA/Ionomycin) or by

solubilizing the cell membrane using non-ionic detergents such as Triton? A Tritiated serotonin release assay (64) was performed to investigate this possibility of an enhanced serotonin secretion by SgIII over-expressing RBL2H3 cells after the above stated stimulations (PMA/ionomycin and triton). The release assay suggested that there was indeed a statistically significant enhancement in serotonin secretion in SgIII over-expressing RBL2H3 mast cells. Unstimulated RBL2H3 cells and SgIII over-expressing RBL2H3 cells in this assay showed no significant evidence of any difference in the leak of ^{3}H serotonin from cytosol. This again supported our general hypothesis that the new granules formed as a result of the over-expression of SgIII could function as *bona fide* secretory granules.

The secretion *versus* degradation theory that arose due to the disappearance and reappearance of SgIII protein in RBL2H3 cells after stimulated with mast cell activators like PMA and ionomycin was analyzed by obtaining total proteins in the supernatant *versus* cells. Western blot analysis of extra-cellular proteins suggested that SgIII protein was exocytosed. Presence of SgIII expression in the supernatants of cells over-expressing SgIII protein in Western blots verified extra-cellular regulated secretion of the protein as would be predicted if SgIII is in the secretory granules.

To study the importance of SgIII protein in mast cells we designed some loss-of-function experiments for which we chose an RNA interference technique, as it is sensitive, fast and specific. We tried to optimize the transfection conditions. Performing transfections with fluorescently labeled siRNA, we realized that RBL2H3 cells were difficult cells for siRNA uptake and transfection. RBL2H3 cells showed no uptake of siRNA at any concentrations tested (5nM, 10nM, 30nM, 100nM). RBL2H3 cells also resisted siRNA uptake even after mimicking one of the published technique (Stevens *et al,* 57) that showed a positive result in siRNA uptake by RBL2H3 cells using different transfection reagent and a different transfection method. We were unsuccessful even when the

RBL2H3 cells were electroporated with siRNA. At this point we figured out two possible reasons for the failure of siRNA uptake in RBL2H3 cells. The first possibility was that the techniques used for siRNA transfections in RBL2H3 cells were not satisfactory and that these cells needed a different strategy for transfections. The second possibility for the failure was that siRNA was entering RBL2H3 cells but failing to knockdown the gene responsible for silencing SgIII protein expression. To test which possibility was true we performed siRNA experiments using the neofection approach in PC12 secretory cells. We observed knockdowns of SgIII in PC12 cells at all concentrations of SgIII siRNA with 100nM concentration of siRNA being the most specific when compared to the non-specific gene suppression, which actually showed no gross changes as compared to the control cells. This ruled out the possibility that the siRNA itself was ineffective suggesting that RBL2H3 mast cells, which were our principal and ideal cells, were simply resistant to siRNA uptake. Although we have knockdowns in the PC12 cells, it is still not specific for SgIII (Figure 24) suggesting that more optimization is required in these cells as well.

At this point we can only suggest the characteristics of SgIII protein in over-expression system that leads to the formation of large secretory granules that seem to behave like mast cell granules. Our observations also suggest that after stimulation with mast cell activators, SgIII protein behaves in a manner similar to mast cell proteases and follows a similar secretory pathway as mast cell proteases. These features suggest that SgIII could actually be a component of mast cell secretory machinery.

Our future approach for loss-of-function experiments shall be to optimize siRNA transfection conditions in RBL cells with other variables. Other alternate approaches of suppressing SgIII expression in mast cells like shRNA approach and the traditional antisense approach should also be considered. Once specific knockdowns are achieved secretion experiments have to be designed to study the effects in the cells lacking SgIII

expression. However, a recent paper by Hendy et al (70) suggests that knockdown of SgIII, even if achieved, may not be very informative. In this paper, the authors show that in CgA knockout mice, the expression levels for all other Granins are elevated 2-3 fold versus littermate controls. This is an apparent compensation mechanism, meaning that it might be necessary to knockout (or knockdown) all the members of the family with overlapping functions in order to see a clear phenotype.

Interestingly there is one published paper on SgIII knockdown mouse (Sutcliffe *et al.*, 68). We contacted Dr. Sutcliffe for the SgIII knockdown mice for our project but unfortunately he replied back saying that the mice no longer existed. This was a shame for us as we could have harvested the SgIII deficient mast cells and compared them *versus* the normal wild type mast cells.

Since the Granin family members have been extensively studied in PC12 cells and we can achieve SgIII knockdowns in these cells, we could design secretion assays on PC12 cells. Comparison of the PC12 (neuroendocrine) secretion in the presence of SgIII and absence of SgIII might unravel some mysteries related to the regulation and function of the protein. We have noted published studies (69) where tyrosine hydroxylase is used as a marker of neuroendocrine secretion in PC12 cells, and we may be able to adopt this assay system.

Finally, we can ask - what is the molecular function of Secretogranin III? This question still remains unanswered, and despite >400 papers being published on Granins, there is no answer to this question for any family member. Some possible answers to this question that offer themselves are that (1) SgIII protein could be a protein that assisted in anchoring other proteins to components of secretory granules. (2) It is possible that SgIII might serve as a messenger by being secreted extra-cellularly. (3) SgIII might function as a chaperone and assist other proteins by binding to them and targeting them to the vesicles in mast cells. (4) lastly it can be proposed that SgIII is actually involved in

secretory vesicle biogenesis in mast cells. Current experiments that are being done are taking a molecular biology approach to dissect the functional domains in SgIII.

Literature Cited.

1. Benyon, R.C. 1989. The human skin mast cell. *Clin Exp Allergy* 19:375-387.

2. Galli, S.J., and S. Nakae. 2003. Mast cells to the defense. *Nat Immunol* 4:1160-1162.

3. Krishnaswamy, G., J. Kelley, D. Johnson, G. Youngberg, W. Stone, S.K. Huang, J. Bieber, and D.S. Chi. 2001. The human mast cell: functions in physiology and disease. *Front Biosci* 6:D1109-1127.

4. Theoharides, T.C. 1996. The mast cell: a neuroimmunoendocrine master player. *Int J Tissue React.* 18:1-21.

5. Maurer, M., T. Theoharides, R.D. Granstein, S.C. Bischoff, J. Bienenstock, B. Henz, P. Kovanen, A.M. Piliponsky, N. Kambe, H. Vliagoftis, F. Levi-Schaffer, M. Metz, Y. Miyachi, D. Befus, P. Forsythe, Y. Kitamura, and S. Galli. 2003. What is the physiological function of mast cells? *Exp Dermatol* 12:886-910.

6. Theoharides, T.C., and D.E. Cochrane. 2004. Critical role of mast cells in inflammatory diseases and the effect of acute stress. *J Neuroimmunol* 146:1-12.

7. Krishnaswamy, G., O. Ajitawi, and D.S. Chi. 2006. The human mast cell: an overview. *Methods Mol Biol* 315:13-34.

8. Metcalfe, D.D., D. Baram, and Y.A. Mekori. 1997. Mast cells. *Physiol Rev* 77:1033-1079.

9. Church, M.K., Y. Okayama, and S. el-Lati. 1991. Mediator secretion from human skin mast cells provoked by immunological and non-immunological stimulation. *Skin Pharmacol* 4:15-24.

10. Sharma, B.B., J.R. Apgar, and F.T. Liu. 2002. Mast cells. Receptors, secretagogues, and signaling. *Clin Rev Allergy Immunol* 22:119-148.

11. Bruhns, P., S. Fremont, and M. Daeron. 2005. Regulation of allergy by Fc receptors. *Curr Opin Immunol* 17:662-669.

12. Conner, E.R., and S.S. Saini. 2005. The immunoglobulin E receptor: expression and regulation. *Curr Allergy Asthma Rep* 5:191-196.

13. Rivera, J. 2002. Molecular adapters in Fc(epsilon)RI signaling and the allergic response. *Curr Opin Immunol* 14:688-693.

14. Turner, H., and J.P. Kinet. 1999. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 402:B24-30.

15. Theoharides, T.C. 2002. Mast cells and stress--a psychoneuroimmunological perspective. *J Clin Psychopharmacol* 22:103-108.

16. Galli, S.J., J. Kalesnikoff, M.A. Grimbaldeston, A.M. Piliponsky, C.M. Williams, and M. Tsai. 2005. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 23:749-786.

17. Theoharides, T.C. 2004. Panic disorder, interstitial cystitis, and mast cells. *J Clin Psychopharmacol* 24:361-364.

18. Theoharides, T.C., and P. Conti. 2004. Mast cells: the Jekyll and Hyde of tumor growth. *Trends Immunol* 25:235-241.

19. Theoharides, T.C., J. Donelan, K. Kandere-Grzybowska, and A. Konstantinidou. 2005. The role of mast cells in migraine pathophysiology. *Brain Res Brain Res Rev* 49:65-76.

20. Mekori, Y.A., and D.D. Metcalfe. 2000. Mast cells in innate immunity. *Immunol Rev* 173:131-140.

21. Trabucchi, E., E. Radaelli, M. Marazzi, D. Foschi, M. Musazzi, A.M. Veronesi, and W. Montorsi. 1988. The role of mast cells in wound healing. *Int J Tissue React* 10:367-372.

22. Noli, C., and A. Miolo. 2001. The mast cell in wound healing. *Vet Dermatol* 12:303-313.

23 Zou Xiang, Mats Block, Carl Löfman, and Gunnar Nilsson. 2001. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography. *J. Allergy Clin. Immunol.* 108 (1): 116-121.

24. Helle, K.B. 2004. The Granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects. *Biol Rev Camb Philos Soc* 79:769-794.

25. Huttner, W.B., and S. Natori. 1995. Regulated secretion. Helper proteins for neuroendocrine secretion. *Curr Biol* 5:242-245.

26. Ozawa, H., and K. Takata. 1995. The Granin family--its role in sorting and secretory granule formation. *Cell Struct Funct* 20:415-420.

27. Taupenot, L., K.L. Harper, and D.T. O'Connor. 2003. The chromogranin-secretoGranin family. *N Engl J Med* 348:1134-1149.

28. Natori, S., and W.B. Huttner. 1994. Peptides derived from the Granins (chromogranins/secretogranins). *Biochimie* 76:277-282.

29. Feldman, S.A., and L.E. Eiden. 2003. The chromogranins: their roles in secretion from neuroendocrine cells and as markers for neuroendocrine neoplasia. *Endocr Pathol* 14:3-23.

30. Kim, T., and Y.P. Loh. 2005. Chromogranin A: a surprising link between granule biogenesis and hypertension. *J Clin Invest* 115:1711-1713.

31. Kim, T., J.H. Tao-Cheng, L.E. Eiden, and Y.P. Loh. 2001. Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. *Cell* 106:499-509.

32. Benedum, U.M., P.A. Baeuerle, D.S. Konecki, R. Frank, J. Powell, J. Mallet, and W.B. Huttner. 1986. The primary structure of bovine chromogranin A: a representative

of a class of acidic secretory proteins common to a variety of peptidergic cells. *Embo J* 5:1495-1502.

33. Benedum, U.M., A. Lamouroux, D.S. Konecki, P. Rosa, A. Hille, P.A. Baeuerle, R. Frank, F. Lottspeich, J. Mallet, and W.B. Huttner. 1987. The primary structure of human secretogranin I (chromogranin B): comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region. *Embo J* 6:1203-1211.

34. Chanat, E., S.W. Pimplikar, J.C. Stinchcombe, and W.B. Huttner. 1991. What the Granins tell us about the formation of secretory granules in neuroendocrine cells. *Cell Biophys* 19:85-91.

35. Chanat, E., U. Weiss, W.B. Huttner, and S.A. Tooze. 1993. Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathways. *Embo J* 12:2159-2168.

36. Natori, S., and W.B. Huttner. 1996. Chromogranin B (secretogranin I) promotes sorting to the regulated secretory pathway of processing intermediates derived from a peptide hormone precursor. *Proc Natl Acad Sci U S A* 93:4431-4436.

37. Huh, Y.H., S.H. Jeon, and S.H. Yoo. 2003. Chromogranin B-induced secretory granule biogenesis: comparison with the similar role of chromogranin A. *J Biol Chem* 278:40581-40589.

38. Yoo, S.H., S.H. You, M.K. Kang, Y.H. Huh, and C.S. Shim. 2002. Localization of secretory granule marker protein chromogranin B in the nucleus. *Ann N Y Acad Sci* 971:345-348.

39. Yoo, S.H., S.H. You, M.K. Kang, Y.H. Huh, C.S. Lee, and C.S. Shim. 2002. Localization of the secretory granule marker protein chromogranin B in the nucleus. Potential role in transcription control. *J Biol Chem* 277:16011-16021.

40. Holthuis, J.C., and G.J. Martens. 1996. The neuroendocrine proteins secretogranin II and III are regionally conserved and coordinately expressed with proopiomelanocortin in Xenopus intermediate pituitary. *J Neurochem* 66:2248-2256.

41. Cozzi, M.G., and A. Zanini. 1988. Secretogranin II is a Ca2+-binding protein. *Cell Biol Int Rep* 12:493.

42. Calegari, F., S. Coco, E. Taverna, M. Bassetti, C. Verderio, N. Corradi, M. Matteoli, and P. Rosa. 1999. A regulated secretory pathway in cultured hippocampal astrocytes. *J Biol Chem* 274:22539-22547.

43. Tschernitz, C., A. Laslop, C. Eiter, S. Kroesen, and H. Winkler. 1995. Biosynthesis of large dense-core vesicles in PC12 cells: effects of depolarization and second messengers on the mRNA levels of their constituents. *Brain Res Mol Brain Res* 31:131-140.

44. Laslop, A., C. Tschernitz, and C. Eiter. 1994. Biosynthesis of proteins of large dense-core vesicles in rat PC12 cells: regulation by forskolin and phorbol ester. *Neuroscience* 59:477-485.

45. Park, H.Y., S.H. So, W.B. Lee, S.H. You, and S.H. Yoo. 2002. Purification, pH-dependent conformational change, aggregation, and secretory granule membrane binding property of secretogranin II (chromogranin C). *Biochemistry* 41:1259-1266.

46. Ottiger, H.P., E.F. Battenberg, A.P. Tsou, F.E. Bloom, and J.G. Sutcliffe. 1990. 1B1075: a brain- and pituitary-specific mRNA that encodes a novel chromogranin/secretogranin-like component of intracellular vesicles. *J Neurosci* 10:3135-3147.

47. Rong, Y.P., F. Liu, L.C. Zeng, W.J. Ma, D.Z. Wei, and Z.G. Han. 2002. Cloning and characterization of a novel human secretory protein: secretogranin III. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 34:411-417.

48. Holthuis, J.C., E.J. Jansen, and G.J. Martens. 1996. Secretogranin III is a sulfated protein undergoing proteolytic processing in the regulated secretory pathway. *J Biol Chem* 271:17755-17760.

49. Hosaka, M., M. Suda, Y. Sakai, T. Izumi, T. Watanabe, and T. Takeuchi. 2004. Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A. *J Biol Chem* 279:3627-3634.

50. Hosaka, M., T. Watanabe, Y. Sakai, T. Kato, and T. Takeuchi. 2005. Interaction between secretogranin III and carboxypeptidase E facilitates prohormone sorting within secretory granules. *J Cell Sci* 118:4785-4795.

51. Hosaka, M., T. Watanabe, Y. Sakai, Y. Uchiyama, and T. Takeuchi. 2002. Identification of a chromoGranin A domain that mediates binding to secretogranin III and targeting to secretory granules in pituitary cells and pancreatic beta-cells. *Mol Biol Cell* 13:3388-3399.

52. Maria Luisa Malosio1,2, Tiziana Giordano1,2, Andrea Laslop1,3 and Jacopo Meldolesi. 2003. Dense-core granules: a specific hallmark of the neuronal / neurosecretory cell phenotype. *Journal of Cell Science* 117: 743-749.

53. Beuret N, Stettler H, Renold A, Rutishauser J, Spiess M. 2004. Expression of regulated secretory proteins is sufficient to generate granule-like structures in constitutively secreting cells. *J Biol Chem.* 279(19):20242-9.

54. Chi EY, Lagunoff D. 1975. Abnormal mast cell granules in the beige (Chediak-Higashi syndrome) mouse. *J Histochem Cytochem.* ; 23(2): 117-22.

55. Spicer SS, Sato A, Vincent R, Eguchi M, Poon KC. 1981. Lysosome enlargement in the Chediak-Higashi syndrome. *Fed Proc.* 40(5):1451-5.

56. Burkhardt JK, Wiebel FA, Hester S, Argon Y. 1993. The giant organelles in beige and Chediak-Higashi fibroblasts are derived from late endosomes and mature lysosomes. *J Exp Med.* 178(6): 1845-56.

57 Lixin li, Yi Yang, Richard L. Stevens. 2002. RasGRP4 regulates the expression of Prostaglandin D2 in human and rat mast cell lines. *J Biol Chem.* 278(7): 4725-4729.

58 Olden K, Pratt RM, Jaworski C, Yamada KM. 1979. Evidence for role of glycoprotein carbohydrates in membrane transport: specific inhibition by tunicamycin. *Proc Natl Acad Sci U S A.* 76(2): 791-5.

59 Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem.* 262(12): 5592-5.

60 Keith P. Mintz, Larry W. Fisher, Wojciech J. Grzesik, Vincent C. Hascall and Ronald J. Midura. 1994. Chlorate-induced inhibition of tyrosine sulfation on bone sialoprotein synthesized by a rat osteoblast-like cell line (UMR 106-01 BSP). *J. Biol. Chem.* 269(7): 4845-4852.

61 Kehoe JW, Maly DJ, Verdugo DE, Armstrong JI, Cook BN, Ouyang YB, Moore KL, Ellman JA, Bertozzi CR. 2002. Tyrosylprotein sulfotransferase inhibitors generated by combinatorial target-guided ligand assembly. *Bioorg Med Chem Lett.* 12(3):329-32.

62 Tamir H, Theoharides TC, Gershon MD, Askenase PW. 1982. Serotonin storage pools in basophil leukemia and mast cells: characterization of two types of serotonin binding protein and radioautographic analysis of the intracellular distribution of [3H] serotonin. *J Cell Biol.* 93(3): 638-47.

63 Scherer LJ, Rossi JJ. 2003. Approaches for the sequence-specific knockdown of mRNA. *Nat Biotechnol.* 21(12): 1457-65.

64 Romanin C, Reinsprecht M, Pecht I, Schindler H. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *Embo J.* (12):3603-8.

65 Orenstein NS, Galli SJ, Dvorak AM, Silbert JE, Dvorak HF. 1978. Sulfated glycosaminoglycans of guinea pig basophilic leukocytes. *J Immunol.* 121(2): 586-92.

66 Jones KL, Stewart RM, Fowler M, Fukuda M, Holcombe RF. 1992. Chediak-Higashi lymphoblastoid cell lines: granule characteristics and expression of lysosome-associated membrane proteins.. *Clin Immunol Immunopathol.* 65(3): 219-26.

67 Stokes AJ, Shimoda LM, Lee JW, Rillero C, Chang YT, Turner H. 2006. Fc-epsilon RI control of Ras via inositol (1,4,5) trisphosphate 3-kinase and inositol tetrakisphosphate. *Cell Signal.* 18(5): 640-51.

68 Dopazo A, Lovenberg TW, Danielson PE, Ottiger HP, Sutcliffe JG. 1993. Primary structure of mouse secretogranin III and its absence from deficient mice. *J Mol Neurosci. Winter*; 4(4):225-33.

69 Grundschober C, Malosio ML, Astolfi L, Giordano T, Nef P, Meldolesi J. 2002. Neurosecretion competence. A comprehensive gene expression program identified in PC12 cells. *J Biol Chem.* 27;277(39):36715-24.

70 Hendy GN, Li T, Girard M, Feldstein RC, Mulay S, Desjardins R, Day R, Karaplis AC, Tremblay ML, Canaff L. 2006. Targeted ablation of the chromogranin A (Chga) gene: normal neuroendocrine dense core secretory granules and increased expression of other Granins. *Mol Endocrinol.* [Epub. ahead of print].