PRELIMINARY STUDY ON THE ROLE OF N-TERMINAL REGULATORY DOMAIN OF SYNTAXIN 4 IN MAST CELL EXOCYTOSIS

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INTRODUCTION

1.1 Background

Mast cells are specialized secretory cells possessing granules and are important in immune and inflammatory responses. Molecular mechanisms of immune cell degranulation is defined as the process of granule or vesicle fusion with the plasma membrane and is accompanied by the release of granule/ vesicle contents to cell exterior which utilizes much of the same conserved basic molecular machinery that drives membrane trafficking in most celle. Mast cells degranulate and release the contents of intracellular secretory granules upon crosslinking of surface FcεRI. A variety of pharmacologically active products like histamine, serotonin, chemotactic factors, immunoregulatory cytokines and other biologically active inflammatory mediators are discharged from activated mast cells. These mediators play central roles in progression of inflammatory (allergic) reactions and also contribute to autoimmune diseases. The major contribution of mast cells in immediate hypersensitivity results from the release of inflammatory mediators in pre- formed granules/ vesicles through a process known as regulated exocytosis via a vesicle fusion mechanism with the plasma membrane. While a great deal is known about the cell surface events that are the source of stimulation, especially regarding allergen/ immunoglobulin E binding to FcεRI receptor and subsequent activation of tyrosine kinases and calcium signalling events that transmit the stimulus to the secretory apparatus, much less is known about the distal mechanisms that regulate the final secretory processes. The transport, fusion and release of vesicle contents through exocytosis are mediated by a family of proteins known as SNAREs (Woska and Gillespie, 2012).

Soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) have been proposed to mediate all intracellular membrane fusion events. These are a family of evolutionary conserved proteins and have been postulated to mediate the fusion of inflammatory mediator-laden vesicles to the membrane in mast cells leading to their subsequent exocytosis. The functional roles of specific SNARE family member complexes in mast cell degranulation have not been fully elucidated. Initially discovered in neuronal cells, the SNARE complex is composed of a vesicle bound v-SNARE protein called VAMP or synaptobrevin and two target organelle t-SNARE proteins, called SNAP-23/SNAP-25 and Syntaxin. Syntaxin is a 35kDa protein containing an N-terminal regulatory domain, a C-terminal SNARE domain and a C-terminal transmembrane domain anchoring it to the plasma membrane. SNAP-23/ SNAP-25 are two complementary isoforms lacking transmembrane domain anchors but localize to plasma membrane due to palmitoylated cysteine residues. VAMP/ Synaptobrevin are proteins lacking a C-terminal transmembrane domain anchoring it to the vesicle membrane (Foster *et al.,* 1998).Individual SNAREs on opposing membranes associate into core complexes via their SNARE motif which consists of homologous coiled coil regions, arranged in heptad repeats and comprised of approximately 60 residues (Fasshauer, 2003). SNARE complex formation is thought to occur after the initial interaction of the N-terminal side of the SNARE proteins followed by N- to C- terminal zippering, thereby bringing membranes into close approximation and initiating membrane fusion. After fusion, SNAREs are recycled via dissociation mediated by the ATP-dependent cofactor NSF (N-ethylmaleimide-sensitive factor) and α-SNAP (soluble N-ethylmalaeimide-sensitive factor attachment protein). For mast cell activation and degranulation occurring in response to aggregated IgE binding to FcεRI, the Qb,c-SNARE SNAP-23 is important, for either granule to surface fusion and/ or inter-granule fusion. Synaptosome-associated protein of 23 kDa (SNAP-23) is central in SNARE complex formation. The Qa- SNAREs Syntaxin 2, Syntaxin 3 and Syntaxin 4 can each form complexes with SNAP-23. The Syntaxin 4-SNAP-23 Q-SNARE complex forms at least three trans-SNARE complexes, one each with VAMP 2, VAMP 3 and VAMP 8 (Pombo *et al.,* 2003). Much work is still required to determine the exact function of these individual trans-SNARE complexes in mast cells.

Syntaxin 4, member of the Qa family, is an exocytotic protein reported in mast cell plasma membrane and is linked to the degranulation process. A role for Syntaxin 4 in regulating vesicular docking and fusion events has also been reported in α- granule secretion of platelets (Flaumehaft *et al.,* 1999), suggesting that Syntaxin 4 is an important t-SNARE in regulated exocytosis of nonneuronal cells. Both Syntaxin 4 and Syntaxin 3 are present in mast cells, and have been shown to be able to form SNARE complexes.

1.2 Current studies

Syntaxin 1 (STX1) has been thoroughly studied and many of its features including its structure have been characterized. Syntaxin 1 is the neuronal iso-form and is anchored to the plasma membrane by a single membrane spanning segment of 23 amino acids (Bennet *et al.* 1992). It is already established that neuronal SM protein Munc18a binds Syntaxin 1A when the latter is in the closed conformation (Misura *et al.,* 2000) and also when STX1A is in the open conformation (via the N-terminal peptide region in STX1A) (Rickman *et al.,* 2007). Like most Syntaxins, Syntaxin 4 contains an independently folded N-terminal domain which is an important regulatory region in these proteins. This domain contains a short N-terminal peptide followed by three-helix bundle called the Habc domain similar to neuronal Syntaxin 1 (Fernandez *et al.,* 1998). Syntaxin 4 binds to the regulatory protein Munc18c involving its N-terminal region. Munc 18c binds to monomeric Syntaxin 4 and the N-terminal 29 amino acids of Syntaxin 4 are necessary for this interaction (Latham *et al.,* 2006). Syntaxin 3 and Syntaxin 4 share 34 % identity in N-terminal region, yet Syntaxin 3 interacts minimally with Munc 18c *in vitro*.

1.3 Research Hypothesis

Syntaxin 4 is structurally similar to its neuronal homologue Syntaxin 1 and it is established that complex domain structure of Syntaxin 1 is required for a multifaceted functional role in synaptic vesicle fusion. However, unlike neuronal STX1, not much is known about regulation of Syntaxin 4 and the functions of various regulatory domains of Syntaxin 4 in orchestration of mast cell exocytosis. So, in the current study, the obtained constructs of wild type Syntaxin 4 and truncated Syntaxin 4 mutant plasmids which lacks the N-terminal regulatory domain are to be recloned into fluorescent molecule tagged cloning vectors that will provide these plasmids a fluorescent tag. On transfecting them into RBL mast cells, the fluorescent tags could be tracked in the cells and study the localization of the protein. The expressed proteins are also readily detectable by western blotting and characterized using specific antibodies and thus the direct effects on mast cell exocytosis could be observed. The importance of Syntaxin 4 and its N-terminal regulatory domain in mast cell exocytosis can thus be unequivocally established.

1.4 Objectives

1.4.1 General objective

- To study the role of N-terminal regulatory domain of t-SNARE Syntaxin 4 in regulated exocytosis from mast cells.

1.4.2 Specific Objectives

- To reclone rat Syntaxin 4 wild type and mutant (N-terminal deleted) constructs into fluorescent tagged EGFP and DsRed vectors.

- To transfect the recloned tagged plasmids into mast cells and study the intracellular/ subcellular localization of Syntaxin 4 in mast cells.

- To study the direct and indirect effects of Syntaxin 4 on mediators release from mast cells.

1.5 Rationale and Scope

Secretory granule/plasma membrane fusion is vital for regulated exocytosis and identification of molecular mechanisms regulating the process helps in identifying ways to modulate exocytosis. SNAREs play a central role in membrane fusion process and investigations of the locations and functions of SNAREs in immune cells such as mast cells is important for both defining immunological pathways and increasing knowledge of the biology and regulation of SNAREs themselves. Elucidating the specific roles of SNAREs in disease or membrane fusion, might be helpful to generate novel targets for therapeutic intervention in adverse inflammatory or allergic diseases. The rationale for the proposed research is that once the mechanisms regulating the function of Syntaxin 4 in mast cell exocytosis are established, it will become possible to modulate the complex for therapeutic purposes, either pharmacologically or through molecular based strategies.

LITERATURE REVIEW

2.1 Membrane Fusion

The eukaryotic cells have a complex intracellular organization and are compartmentalized into distinct membrane bound organelles. Cellular life and differentiation depend on keeping the integrity of the boundaries of a cell and its organelles intact at all times (Jahn, 2004). The plasma membrane of a cell which is a structured bilayer of phospholipids, cholesterol and protein molecules serve as a barrier between the cell and the extracellular environment. Selective permeability of the membrane only involves the transfer of selective (lipophilic) molecules. A number of proteins are synthesized in cells that need to be transported to their appropriate destinations in the cell or outside of it. The membrane impermeable macromolecules (membrane constituents and cargo) need to be delivered to their correct intracellular destinations without compromising the structural integrity. Molecules synthesized can be targeted to the inner space of an organelle, different intracellular membranes, plasma membrane, or to exterior of the cell via secretion. Furthermore, organelles need to be generated continuously during growth and cell division and to achieve this, eukaryotic organisms have developed elaborate mechanisms that execute the budding, splitting and fusion of organelles and whole cells without leakage of intracellular content or disturbing the surrounding membrane (Bonifacino and Glick, 2004).

In the intracellular membrane trafficking, the key event is membrane fusion and this is the most fundamental process in life. Membrane fusion occurs when two separate lipid membranes come together and merge into a single continuous bilayer (Jahn *et al.*, 2003). Fusion can be homotypic occurring when similar compartments fuse or heterotypic when different compartments fuse (Chernomordik and Kozlov, 2005). The mechanism of membrane fusion is thought to occur via an intermediate stage before complete fusion. This intermediate stage known as hemifusion, occurs when the apposing monolayers merge. After hemifusion, a fusion stalk is formed which when develops into a fusion pore, result in the complete membrane fusion of the two bialyers (Chernomordik and Kozlov, 2005). The membrane enclosed organelles of eukaryotic cells communicate with each other through the exchange of membrane bound carriers, commonly referred to as vesicles. Once a vesicle is generated from a precursor membrane, it is transported to its destination and finally fusion of the vesicle occurs with the target compartment (Jahn *et al.*, 2006). The carrier vesicles are loaded with soluble and membrane bound cargo, bud off a donor compartment and move to, then dock and fuse with a target membrane to deliver their cargo. The proper function of vesicle targeting and fusion is crucial for maintenance of cellular integrity, normal growth and for intracellular signaling events. Carrier vesicles are generated for each successive step of anterograde and retrograde transport between cell compartments in endocytic or exocytic pathways (Stow *et al.*, 2006).The endocytic and exocytic systems are the two trafficking networks that enable cells to communicate with their surroundings. Both processes directly impact in the cell biosynthesis and also in cell signaling. Endocytosis allows uptake of impermeable molecules such as cholesterol or iron ions and allows the regulated internalization of receptors into peripheral early endosomes, thereby modulating responses to external stimuli (Sagi-Einsberg, 2007). Exocytosis or the secretory pathway can be defined as the fusion of an intracellular trafficking vesicle with the plasma membrane. Exocytosis enables the incorporation of proteins and lipids in the plasma membrane and is essential for growth and differentiation of a cell. As shown in Figure 2.1, the eukaryotic cells undergo constitutive exocytosis which includes all fusion events in which vesicles are generated, transported and exocytose continuously. However, the specialized secretory cells such as neurons, cells of the endocrine, exocrine and immune systems show regulated secretion. Regulated exocytosis allows for the controlled delivery of secretory molecules that are stored in specialized intracellular pools and gets mobilized upon the activation of signaling cascades (Jahn, 2004). Exocytosis at the molecular level, involves specialized protein families that are conserved throughout eukaryotic evolution from yeast to humans.

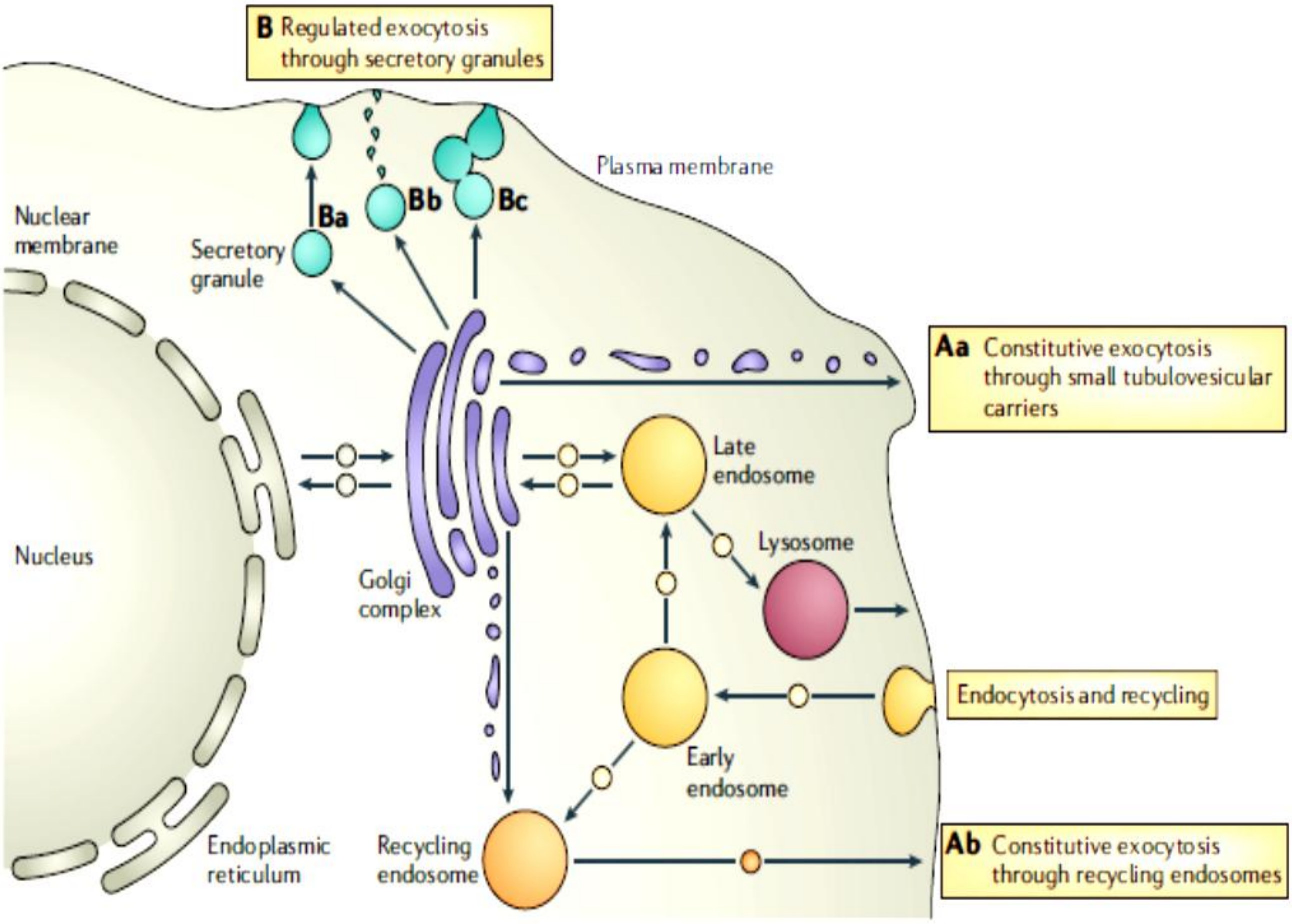

Figure 2.1: Intracellular trafficking pathways. In all the eukaryotic cells, there are complex pathways for the transport of proteins and membranes between cell organelles, and to and from the cell surface in small carrier vesicles. Constitutive exocytosis occurs through small carriers going directly to the cell surface (Aa) or through the recycling endosomes (Ab). In addition, some immune systems also have secretory granules for regulated exocytosis (B), where secretory granules can fuse directly with the plasma membrane for release (Ba). The other variations include the transport of cargo to the cell surface in small vesicles, known as piecemeal exocytosis (Bb), or release by fusion of granules with each other at the cell surface, known as compound exocytosis (Bc) (Stow *et al.*, 2006).

2.2 SNAREs: Mediators of membrane fusion

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins are thought to play a key role in the intracellular membrane fusion events (Rothman, 1994). Membrane fusion is not a simple process to achieve since the energy barrier resulting from the close apposition of two membranes needs to be overcome. The main candidate proteins helping to overcome this energy barrier are the SNAREs, which are assisted by tethering factors and other regulatory molecules (Xu *et al.*, 2005). The association of SNAREs is believed to encompass functionally relevant step in membrane fusion, and for the fusion to occur, complementary set of SNAREs need to be present on the two membranes. It is postulated that each type of transport vesicle has a distinct v-SNARE that pairs with a unique cognate t-SNARE at the appropriate target membrane, and that this specific interaction mediates the vesicles fusion at the correct membrane (Jahn, 2004). SNAREs were originally divided into v-SNARES and t-SNAREs according to their vesicle or target membrane localization (Sollner *et al.*, 1993). However, this terminology was not useful in the case of homotypic membrane fusion events and certain SNAREs function in several transport steps with varying partners. SNAREs then have been reclassified based on the identity of a highly conserved residue as R-SNAREs (arginine- containing SNAREs) or Q-SNAREs (glutamine- containing SNAREs) (Sutton *et al.,* 1998).

SNARE motifs, an evolutionarily conserved stretch of 60-70 amino acids arranged in heptad repeats, mediate complex formation and are associated with conformational and free energy changes. SNAREs when monomeric, SNARE motifs are unstructured and when appropriate sets of SNAREs are combined, the SNARE motifs spontaneously associate to form helical core complexes of extraordinary stability (Fasshauer, 2003). The stable core complex is resistant to SDS denaturation, protease digestion and clostridial neurotoxin cleavage and is heat stable upto 90^{o} C (Yang *et al.,*1999).Interaction between v-SNARE and t-SNARE leads to the formation of the trans-SNARE complex (or SNAREpin), in which the four SNARE motifs assemble as a twisted parallel four-helical bundle (Figure 2.2) (Hong, 2005). The formation of this bundle leads to a tight connection of the membranes that are destined to fuse, and initiates membrane fusion (Jahn and Scheller, 2006). Core complexes are represented by elongated coiled coils of four intertwined, parallel α- helices, with each helix being provided by a different SNARE motif. The centre of the bundle consists of 16 stacked layers of interacting side chains and these layers are largely hydrophobic. The central '0' layer contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue.

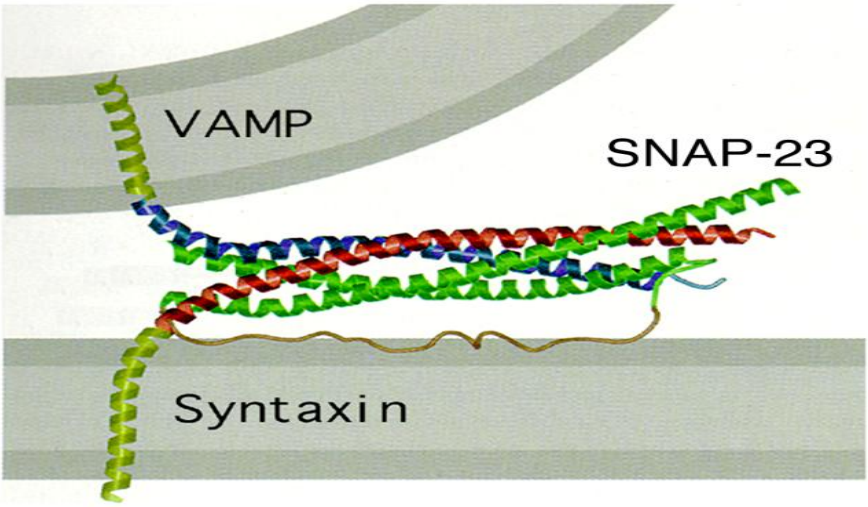

Figure 2.2: Hypothetical model of the synaptic fusion complex. Based on the X-ray crystal structure at 2.4 Å resolution, crystal structure of synaptic trans-SNARE complex containing STX 1A, VAMP 2 and SNAP-25/23 (Sutton *et al.,* 1998).

On the basis of this, the contributing SNARE motifs are classified into Qa-, Qb-, Qc- and R- SNAREs (shown in Table 1) and each of this is required to form hetero-oligomeric, parallel four helix bundle of functional SNARE complexes that drive membrane fusion (Fasshauer *et al*., 1998). A t-SNARE is generally assembled from one heavy chain and two light chains of SNARE domains. The two light chains can come from one or two proteins. The Syntaxin (STX) subfamily contributes one SNARE motif as the t-SNARE heavy chain, and a member of the SNAP-25 subfamily contributes two SNARE motifs as two t-SNARE light chains (Hong, 2005). SNAREs are usually small proteins of around 100-300 amino acids in length and consist of an evolutionally conserved SNARE motif of about 60 residues in all known SNAREs. They have a simple domain structure and at their C-terminal ends, most SNAREs have a single transmembrane domain that is connected to the SNARE motif by a short linker. Most SNAREs are also characterized by an extended N-terminal domain with coiled-coil regions.

SNARE proteins have many common structural features. The majority of SNARE proteins have a C-terminal membrane spanning domain, an N-terminal domain and a SNARE motif which consists of heptads repeats of approximately 60-70 amino acids (Figure 2.3). This region is common to all SNAREs and is critical for SNARE complex formation and for fusion specificity (Paumet *et al.*, 2004). Although this prototypic structure applies to most SNAREs, a subset of SNAREs (including the evolutionarily younger brevins) lacks the N-terminal domains. The SNARE subgroup, SNAP-25 family contains hydrophobic post-translational modifications that mediate membrane anchorage and contains two different SNARE motifs joined by a palmitoylated flexible linker (Rossi *et al.,* 2004).

Recent studies have shown that SNAREs carrying transmembrane domains can also be palmitoylated and it protects SNAREs from ubiquitylation and subsequent degradation (Valdez-Taubas and Pelham, 2005). The N-terminal domains of the three- helix bundle type reversibly associate with the SNARE motif of the same SNARE to form a closed conformation, which prevents the SNARE motif from forming a SNARE complex (Dulubova *et al.,* 1999). The N-terminal domains also function as recruitment platforms for the binding of other regulatory proteins like SM (Sec1/ Munc18- related) proteins. A general comparison of overall structure of SNARE proteins is shown in Figure 2.4.

Table 1: Functional classification of SNAREs (Hong, 2005)

Basis of classification	v-SNARE	t-SNARE
Sub-classification of t-SNARE	Heavy chain and light chain	
Structurally according to central residue of SNARE motif (O layer)	R-SNARE	Q-SNARE
Sub-classification of Q-SNARE	Qa, Qb and Qc	

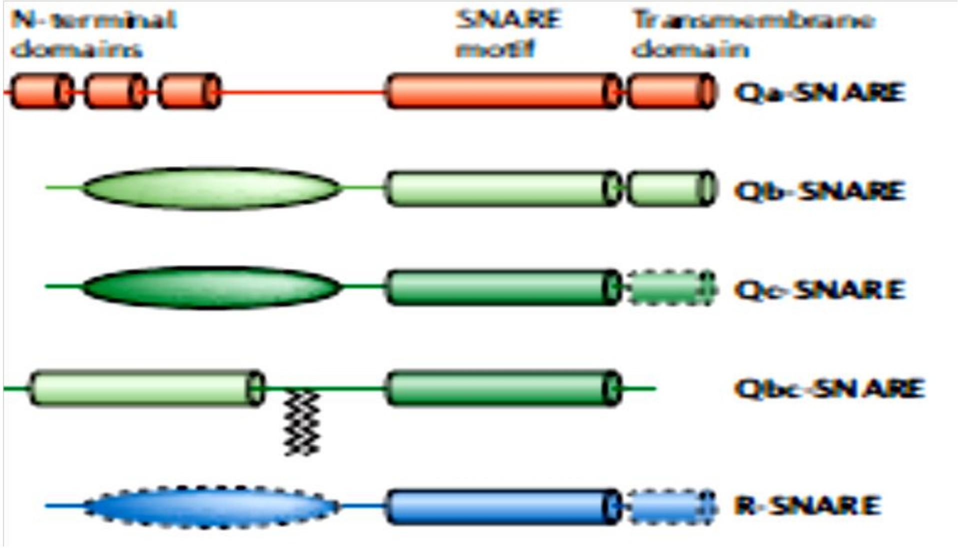

Figure 2.3: Structure of SNAREs. The domain structure of Qa-SNAREs have N-terminal antiparallel three-helix bundles and other N-terminal domains represented by a basic oval shape. The domains missing in some subfamily are represented by dashed domain borders. The SNAP-25 subfamily contains one Qb- SNARE motif and one Qc- SNARE motif and these motifs are connected by a linker that is frequently palmitoylated (zig-zag lines) (Jahn and Scheller, 2006).

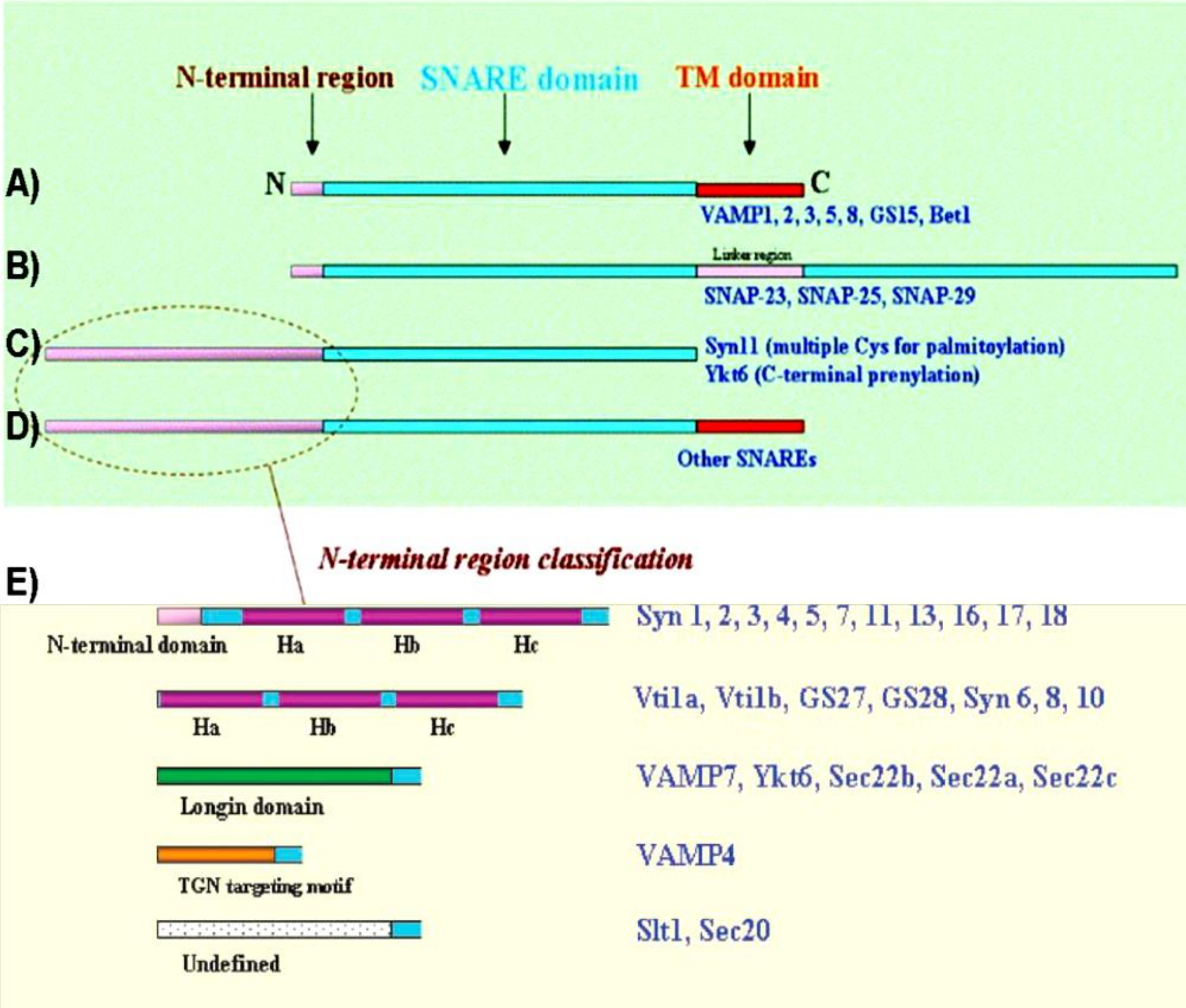

Figure 2.4: General comparision between domains of different SNARE proteins. A) Proteins from the VAMP family have a short N-terminal region, one SNARE domain and a C-terminal trans-membrane domain which anchors VAMP to the vesicle membrane (red). **(B)** Members from the SNAP-25 family are composed of two SNARE domains connected by a linker that is post-translationally modified by addition of palmitate groups to conserved central cysteine residues. **(C)** Some SNAREs that do not possess trans-membrane domains (Syntaxin 11 and Yky6); **(D)** Most of Syntaxins contain a C-terminal trans-membrane region (red), a SNARE motif (light blue) and a N-terminal domain which is autonomously folded (pink). **(E)** Comparisons between the N-terminal domains of different SNARE proteins (Hong, 2005).

2.3 Different locations of SNARE proteins

SNARE proteins form a super family of small proteins with 25 members in *Saccharomyces cerevisiae*, 36 members in humans and 54 members in *Arabidopsis thaliana* (Jahn, 2004). Of the known mammalian SNAREs, based on their sequence homology and domain structure, they have been categorized as members of Syntaxin, VAMP or SNAP-25 families. The synaptic proteins Syntaxin (STX1), SNAP-25 (synaptosome-associated protein of 25 kDa) and VAMP (vesicle-associated membrane protein, also called synaptobrevin) were the first SNAREs to be discovered. The Syntaxin and VAMP both are anchored to the membrane via carboxy-terminal transmembrane domain while SNAP-25 is attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein (Chen and Scheller, 2001). SNAREs in order to function in specific intracellular fusion steps, there must be sorting mechanisms ensuring that there are appropriate set of SNARE proteins in each intracellular membrane (Jahn and Scheller, 2006).

Many SNAREs reside predominantly, or even selectively, in specific subcellular compartments (Figure 2.5). As for example, Syntaxin 1, 2, 3, 4, SNAP-23 and SNAP-25 are present at the plasma membrane, VAMP on synaptic and neurosecretory vesicles, and Syntaxin 5 and VAMP 4 in the Golgi apparatus (Hong, 2005). In all heterotypic fusions, after completion of most fusion reactions of small trafficking vesicles with a large acceptor compartment, some SNAREs need to be returned to their donor compartment by intracellular membrane trafficking. So, SNAREs not only reside in the organelle for which they mediate fusion, but also in the membranes of the organelles that are part of their recycling pathway. Some other SNAREs have an even more widespread distribution, including those in endosomal trafficking (Antonin *et al.,* 2002). Therefore, localization is dependent on the steady state between SNARE biosynthesis, fusion and recycling.

2.3.1 Sorting and Localization of SNAREs inside cell

If SNAREs function in all cellular fusion reactions, they are expected to be present in the membranes of all intracellular compartments. Indeed, SNAREs are widely distributed in cells, and each SNARE exhibits a characteristic subcellular distribution SNAREs are often associated with transport vesicles and shuttle between trafficking compartments (Jahn *et al.*, 2003). As a result, many SNAREs are present on multiple intracellular compartments. Therefore, it is not sufficient to localize SNARE in order to determine its site of action. For instance, SNAP-25 and Syntaxin are distributed over the entire neuronal plasma membrane in adult animals, including myelinated axons. Nevertheless, in mature neurons, these SNAREs are only known to function at the synapse, a location that contains a small minority of the total pool of these SNAREs (Zerial & McBride, 2001). SNARE proteins are sorted to their intracellular destinations by unknown mechanisms. Synaptobrevin (and probably also other SNAREs containing C-terminal transmembrane regions) is post-translationally inserted into the membrane of the endoplasmic reticulum and then transported to the synapse (Metcalfe *et al.*, 1997). Insertion is ATP dependent and requires proteins different from the translocation complex needed for proteins with a genuine signal sequence (McNew *et al.*, 2000). Sorting of membrane-associated SNAREs probably involves cytoplasmic sequences and transmembrane regions for those SNAREs. However, mutations in the SNARE motifs disturb SNARE complex assembly or disassembly which may result in mislocalizations that are unrelated to protein sorting.

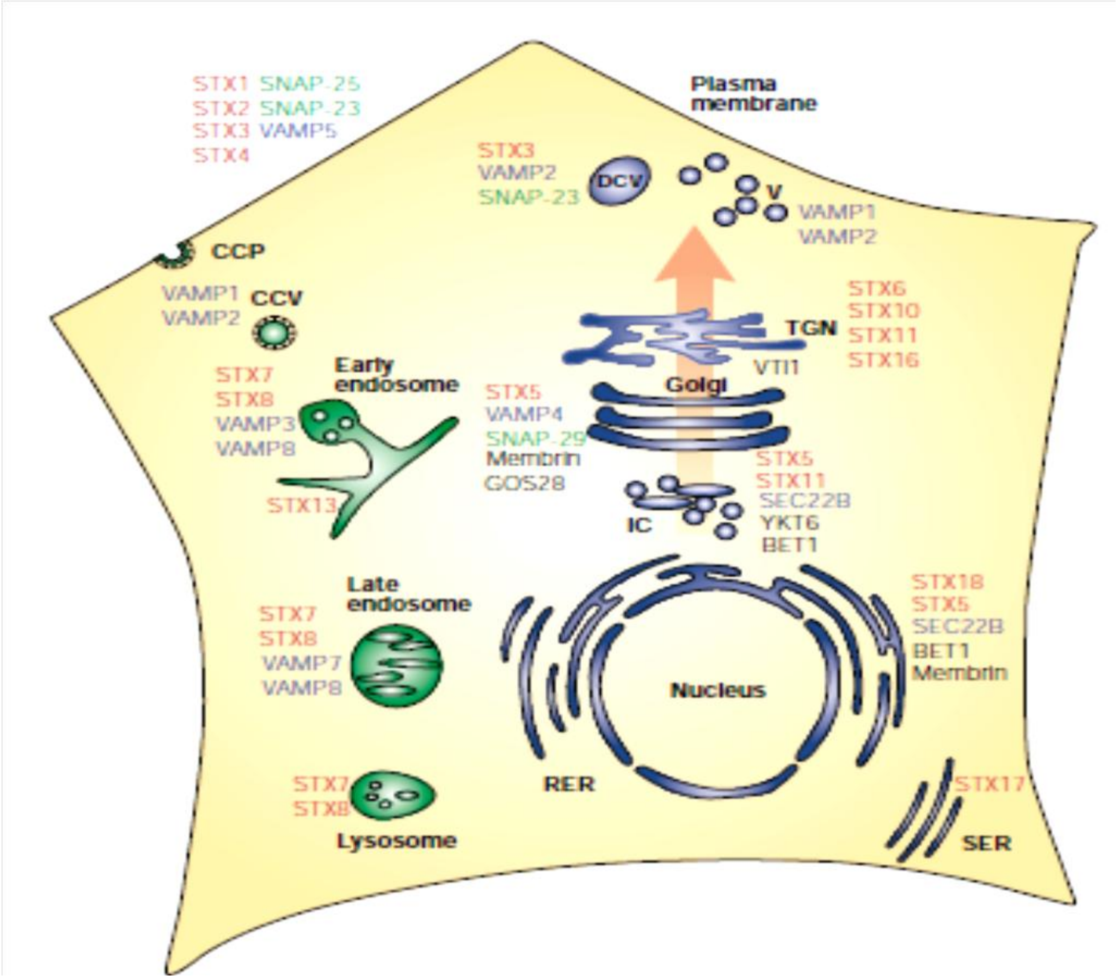

Figure 2.5: Subcellular localization of mammalian SNAREs within the cell. SNARE proteins are usually found in specific cellular compartments within the cell which indicates that their localization might influence in their function and specificity in membrane trafficking. The SNARE proteins can be found at the plasma membrane (facing the cytosol), or on intracellular vesicles and organelles (Chen and Scheller, 2001).

2.4 Syntaxin Family

Syntaxins were first identified as 35 kDa proteins present in the nervous system concentrated to the plasma membrane (PM) of pre-synaptic neurons (Bennett *et al.*, 1992), but since then many homologues have been identified in many phyla. There are currently 15 syntaxins in mammals and 8 in yeast which localise to different intracellular compartments and are cytoplasmically oriented (Hong, 2005; Teng *et al.*, 2001). Apart from this C-terminal domain, syntaxins contain a SNARE motif and an N-terminal domain. This N-terminal region is conserved in all isoforms which are localised at the PM such as Syntaxins 1A, 2, 3 and 4 (Bennett *et al.*, 1992). In the Syntaxin subfamily, the N-terminal domain consists of an antiparallel bundle of three α-helices, Habc-domain that fold back onto the SNARE motif to produce the closed conformation (Dulubova *et al.*, 1999).

2.5 SNAP-23/25 Family

The first SNARE protein from the SNAP family to be characterized was SNAP-25 which is present in neurons and neuroendocrine cells (Oyler *et al.*, 1989). Later, three more members of the SNAP-25 family were identified and described as SNAP-23, SNAP-29 (Steegmaier *et al.,* 1998) and SNAP-47 (Holt *et al.,* 2006) which unlike SNAP-25, are ubiquitously expressed (Ravichandran *et al.*, 1996). These SNARE proteins are thought to be targeted to membranes post-translational and most of them are peripheral membrane proteins. SNAREs like SNAP-25, 23 and 29 do not contain trans-membrane domains and, unlike VAMP and Syntaxins, contain two SNARE motifs, which participate in the formation of the SNARE core complex. SNAP-25 and SNAP-23 were shown to be palmitoylated *in vivo* at cysteine residues located in the linker region which connects their two coiled-coil domains helping these proteins to attach to membranes (Lane and Liu, 1997; Vogel and Roche, 1999). SNAP-29, unlike SNAP-25 and SNAP-23 is not palmitoylated and associates with membranes *via* its direct interaction with syntaxins (Steegmaier *et al.*, 1998).

2.6 VAMP Family

VAMP (vesicle associated membrane proteins) or synaptobrevins were first described as integral membrane components of synaptic vesicles (Trimble *et al.*, 1988). They are small conserved proteins of ~120 amino acids which contain a proline-rich amino terminus, a highly charged internal region, and a hydrophobic carboxyl-terminal domain (trans-membrane domain) which anchors the protein to the membrane and, like the syntaxin family, a SNARE motif of ~70 amino acids (Trimble *et al.*, 1988). The mammalian VAMP subfamily contains seven members: VAMP 1 (synaptobrevin 1), VAMP 2 (synaptobrevin 2), VAMP 3 (cellubrevin), VAMP 4, VAMP 5 (myobrevin), VAMP 7 (tetanus toxin insensitive), and VAMP 8 (endobrevin) (Hong, 2005). VAMP 1 is present primarily in neurons, whereas other VAMP isoforms are more ubiquitously expressed (Lin and Scheller, 2000).

2.7 SNARE Hypothesis

The SNARE hypothesis, proposed in 1993, was the first working model to explain vesicle docking and fusion in molecular terms (Sollner *et al.*, 1993). This hypothesis postulated that each type of transport vesicle has a distinct v-SNARE that pairs up with a cognate t-SNARE at the appropriate target membrane in antiparallel fashion, and this specific interaction docks the vesicles at the correct membrane (Figure 2.6). Subsequently the dissociation of the SNARE complex occurs by the ATPase activity of NSF driving membrane fusion. The specificity of SNARE pairing dictates the specificity of membrane recognition (Sollner *et al.*, 1993). Although this hypothesis has been widely accepted in the field, some studies have suggested that membrane docking can occur in the absence of SNAREs (Gagescu, 2000). However, reconstitution of yeast SNAREs into liposomes in several possible combinations have confirmed the specificity in SNARE pairing and fusion (McNew *et al.*, 2000).

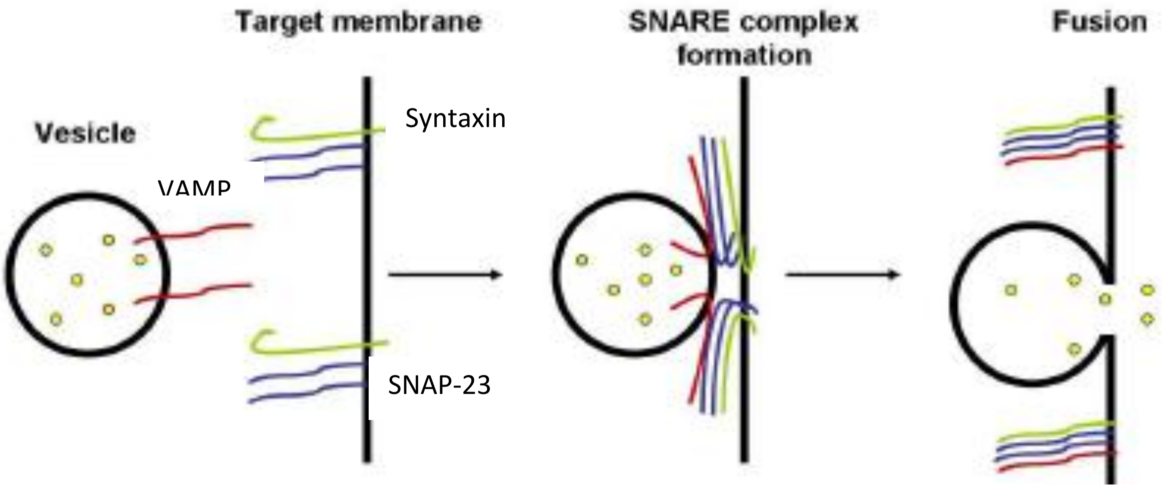

Figure 2.6: Schematic representation of the SNARE hypothesis. The SNAREs, v- on the vesicle and t- on the target come into close proximity which favors the formation of SNARE complexes that will eventually drive membrane fusion by overcoming the energy barrier for fusion (McNew *et al.*, 2000).

2.8 Mode of action of SNARE

SNAREs when discovered as the targets of NSF (N-ethylmaleimide- sensitive factor), there was hypothesis that the fusion is mediated by the action of NSF on the pre-assembled SNARE docking complexes (Sollner *et al.,* 1993). Alternatively some also proposed that SNARE assembly might directly lead to fusion. Later on, it was realized that NSF is not involved in fusion itself, rather SNARE disassembly might be the driving force behind fusion (Hanson *et al.,* 1997). According to this, the zippering of the SNARE motifs from their N-terminal ends towards their C-terminal membrane anchors, clamps the membrane together and initiates fusion where SNAREs directly act as fusion catalysts and the activity of NSF generate uncomplexed, free SNAREs constantly (Jahn and Scheller, 2006). NSF mediates the SNARE priming.

In a general mode of SNARE action (summarized in Figure 2.7), firstly the v-SNARE is packaged together with other cargo proteins into the budding vesicle so that the resulting transport intermediate is competent to fuse with the target compartment. Next during the tethering event mediated by various tethering factors, vesicles are positioned precisely at the region of the target compartment where the t-SNAREs are located (Hong, 2005). The tethering factors, which act over a longer distance than the SNAREs, interact with both the vesicle and the target compartment to facilitate the subsequent pairing of the v-SNARE with the cognate t-SNARE (Shorter *et al.,* 2002). In the third stage, the interaction of v-SNAREs and t-SNAREs on the two opposing membranes mediate the short-range docking of the vesicle with the target compartment followed by the formation of a trans-SNARE complex (Chen and Scheller, 2001). Before complex assembly, the SNARE motifs are believe to be unstructured and become highly organized into a four-helical bundle during the formation of the trans-SNARE complex. The energy barrier for membrane opposition created largely by the negative charges of phoshpholipid head groups of the lipid bilayers may be overcome by the energy released during the SNARE complex assembly. It functions like a zipper and the zippering starts from the N-terminal side and progresses towards the C-terminal end (Sudhof, 2004).

Finally, for the subsequent rounds of transport, the cis-SNARE complex is disassembled which is catalyzed by the combined action of α-SNAP (soluble N-ethylmalemide-sensitive factor attachment protein) and NSF (N-ethylmaleimide- sensitive factor). The interaction of ATPase, NSF (in the form of hexamer) and three α-SNAPs with the cis-SNARE complex leads to the formation of a transient 20 S complex. NSF mediates ATP hydrolysis and disassembles the 20 S complex and the cis-SNARE complex as well. Then the freed v-SNAREs are recycled to the donor compartment by retrograde transport, while the t-

SNARE subunits are re-organized into functional t-SNAREs for the next round of docking and fusion events (Brunger *et al.*, 2003).

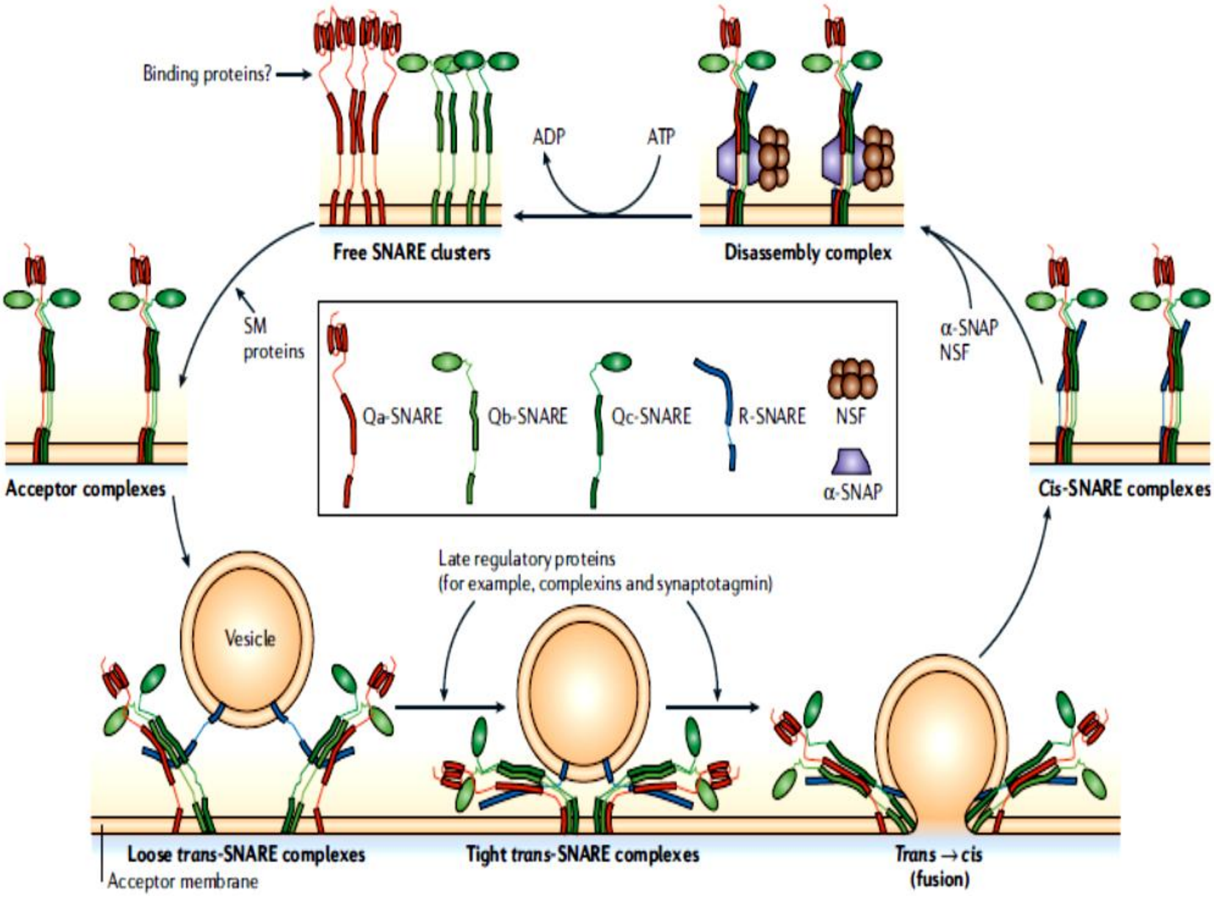

Figure 2.7: SNARE conformational cycle during vesicle docking and fusion. Q-SNAREs organized in clusters (top left), assemble into acceptor complexes, requiring SM (Sec1/Munc18-related) proteins. Acceptor complexes interact with the vesicular R-SNAREs through the N-terminal end of the SNARE motifs, and this nucleates the formation of a four-helical trans complex. Trans-complexes proceed from a loose state (in which only the N-terminal portion of the SNARE motifs are 'zipped up') to a tight state (in which the zippering process is mostly completed), and this is followed by the opening of the fusion pore. In regulated exocytosis, these transition states are controlled by late regulatory proteins that include complexins (small proteins that bind to the surface of SNARE complexes) and synaptotagmin (which is activated by an influx of calcium). During fusion, the strained trans-complex relaxes into a cis-configuration. Cis-complexes are disassembled by the AAA+ protein NSF together with SNAP functioning as cofactors (Jahn and Scheller, 2006).

2.9 Mast cells

Mast cells are tissue localized cells that upon activation release a whole variety of inflammatory mediators. Mast cell precursors which are formed in bone marrow by hematopoiesis; are released into the blood as undifferentiated cells and they do not differentiate until they leave the blood and enter the tissues. Mast cells can be found in a wide variety of tissues, including the skin, connective tissues of various organs and mucosal epithelial tissue of respiratory, genitourinary and digestive tract. Mast cells play an important role in the development of allergies and anaphylaxis, they play an important protective role as well, being intimately involved in wound healing and defense against pathogens (Metcalfe *et al.,* 1997).

Mast cells are specialized secretory cells responding to inflammatory signals and release histamine, serotonin, proteases and other inflammatory mediators upon activation which are stored in the cytoplasmic granules, ensuring an immediate and maximal biologic effect (Blank, 2011). Mast cells are regarded as critical effectors cells in the inflammatory reaction underlying immediate hypersensitivity. MCs produce three categories of effector molecules (Figure 2.8): those which are stored in granules such as serotonin, histamine, heparin, tryptase and chymase; those which are synthesized *de novo* upon cell stimulation such as lipid mediators (PAF), prostaglandins (PDG2) and leucotrienes (LTB4, LTD4); and a large variety of cytokines such as interleukin (IL)-1, IL-3, IL-5, IL-8, IL-10, granulocyte–macrophage-colony-stimulating factor, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) (Wasiuk *et al.,* 2009).

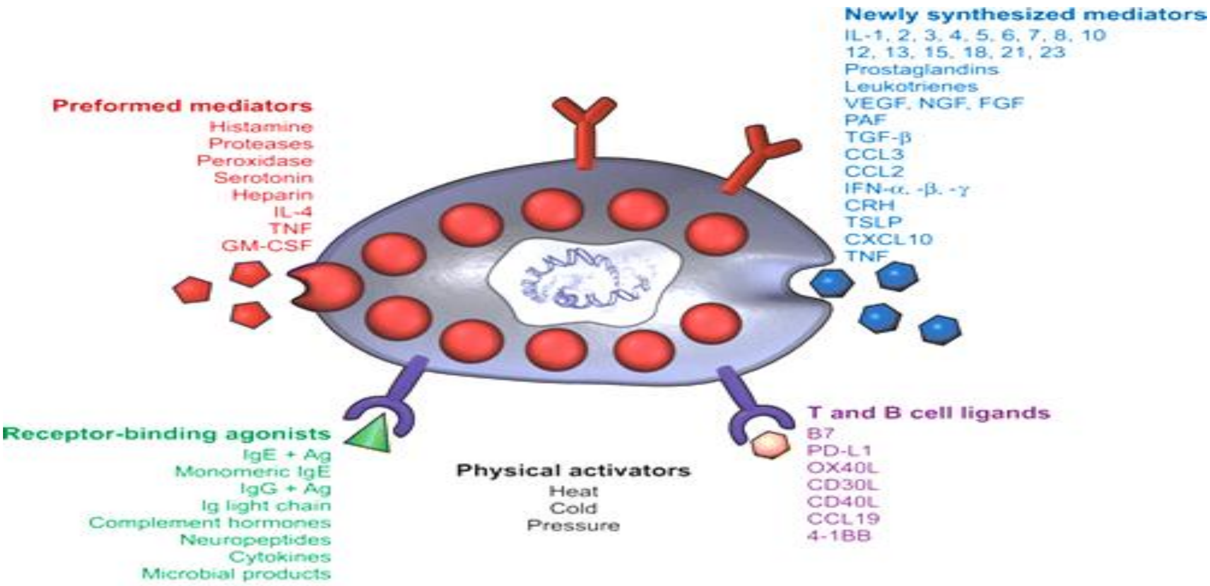

Figure 2.8: Common mast cell activators and secreted mediators (Sayed *et al.,* 2008).

2.10 Mast cell exocytosis

Most of the eukaryotic cells transport and exocytose proteins constitutively while the specialized secretory cells like MCs are capable of regulated exocytosis. Regulate exocytosis is a process in which translocation, docking and fusion of secretory granules with plasma membrane occurs after a trigger or stimulus. The stored mediators from the inflammatory cells are released by at least four modes of exocytosis –classical, compound, piecemeal degranulation and cytolysis (Figure 2.9) (Logan *et al.,* 2003). Classical exocytosis involves the extrusion of single secretory granules after stimulation to the cell exterior by discrete fusion events while compound exocytosis is where multiple intracellular granule-granule fusions lead to secretion from a single fusion pore onto the target surface at the site of cell adherence (Moqbel and Lacy, 1999). In piecemeal degranulation (PMD), there is presence of intact cytoplasmic crystalloid granules containing partially eroded core components and there is selective release of granule derived mediators (Lacy *et al*., 2001). Cytolysis or necrosis or total granule extrusion involves the deposition of intact granules in the tissue coinciding with cell death. Mast cells exhibit compound exocytosis and also utilize piecemeal degranulation (Dvorak, 1997). Progressive secretory granule empyting also referred to as “kiss and run” fusion occurs through a relative and transient formation of fusion pore. During kiss and run, the vesicle membrane does not collapse into the plasma membrane, maintaining the secretory vesicle’s position, identity and potentiality to perform a second round of exocytosis.

Figure 2.9: The different modes of exocytosis in mast cells. Generally upon stimulation, mast cell undergo compound and /or multivesicular (sequential) exocytosis. Another mode of exocytosis, piecemeal degranulation can also be observed with some stimuli involving the gradual emptying of vesicular contents without any evidences for fusion (Blank, 2011).

2.11 Mast Cell activation

Mast cells have fundamental roles in the innate and acquired immune responses through the secretion of the contents of preformed cytoplasmic secretory granules (SGs) on encountering certain stimulants. Though mast cells can be activated by various different mechanisms, the best studied mechanism by which mast cells accomplish immunologically specific function is through antigen and IgE-dependent aggregation of high affinity IgE receptor (FcεRI) (Blank and Rivera, 2004). FcεRI is expressed on the surface of mast cells as a multisubunit (hetero-tetrameric) receptor composed of a ligand binding α-subunit, a signal amplifying membrane-tetra-spanning β-subunit and two homodimeric disulfide-linked γ–subunit. The signaling pathway (Figure 2.10) is initiated through the immunoreceptor tyrosine-based activation motif (ITAM) which is encoded in the cytoplasmic tails of the β and γ subunits. Mast cells express several Src family protein tyrosine kinases (PTKs). A high affinity allergen or aggregation of large numbers of FcεRI induces robust cellular signals that can effectively induce mast cell effector responses. IgE-induced cross linking of cell surface FcεRI activates the kinase Lyn, which phosphorylates FcεRI ITAMs and activates the kinase Syk after ITAM binding. Lyn and Syk phosphorylates several adaptor molecules and enzymes to initiate the cascade of signal transduction.

In addition to activating Lyn, FcεRI aggregation activates a second Src family kinase, Fyn that phosphorylates the adaptor Gab2 to activate the phosphatidylinositol-3-OH kinase (PI3K) pathway. In addition to its aforementioned signal-initiating activity, Lyn also negatively regulates FcεRI-induced signaling events including Fyn activation (Rivera and Gilfillan, 2006). Phospholipase C-γ hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP2) to form soluble inositol-1,4,5-trisphosphate(IP3) and membrane-bound diacylglycerol (DAG). The binding of inositol-1,4,5-trisphosphate to its receptor, a ligand gated selective ion channel in the endoplasmic reticulum membrane, rapidly causes the release of Ca^{++} from endoplasmic reticulum stores. This Ca^{++} mobilization from ER in turn induces prolonged influx of Ca^{++} through store-operated calcium release–activated calcium (CRAC) channels located in the plasma membrane and hence degranulation in mast cells (Rivera and Gilfillan, 2006).

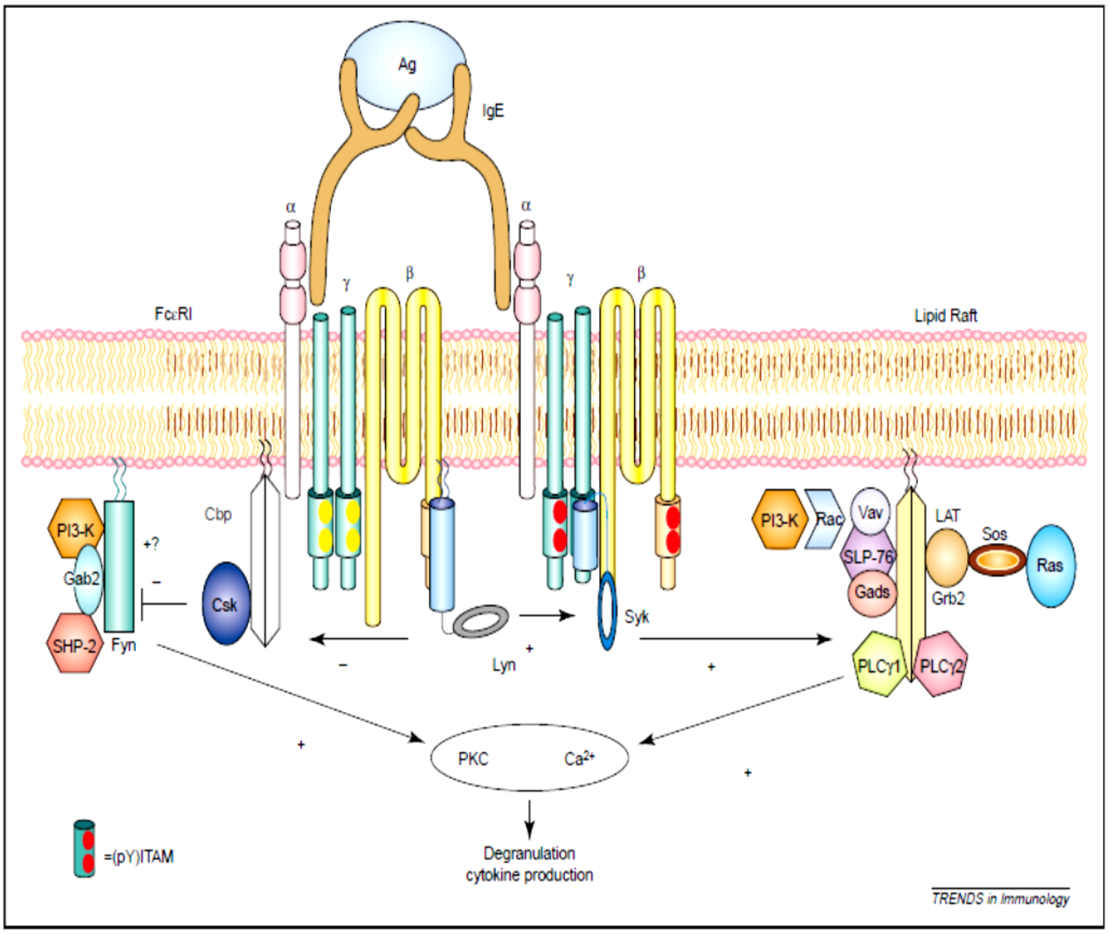

Figure 2.10: Early signaling events in coupling of the high affinity receptor for immunoglobin E(FcεRI). Antigen-aggregation of IgE-occupied FcεRI results in phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) by Lyn kinase (Lyn), activation of Syk kinase (Syk) through ITAM binding, and inclusion of the FcεRI in lipid rafts. Aggregation of FcεRI also rapidly activates Fyn kinase (Fyn), whose activity is important for phosphorylation of the adaptor known as Grb-2 associated binder-like protein 2 (Gab2) and activation of phosphoinositide 3-kinase (PI3-K) activity in mast cells (MCs). Both Fyn and Lyn co-immunoprecipitate with FcεRI, however, it is unclear whether Fyn directly or indirectly interacts with FcεRI, although Lyn is known to directly interact with the tretraspanning β chain. Lyn kinase negatively regulates the activity of Fyn but positively regulates the activation of Syk and phosphorylation of another adaptor protein, linker for activation of T cells (LAT), which scaffolds a complex required for normal MC calcium responses. Fyn kinase positively regulates Gab2 and its association with PI3-K and is essential for initiation of the degranulation response. Signals generated by the two distinct adaptor complexes (LAT and Gab2) synergize to support novel PKC activation (Fyn–Gab2–PI3-K) and calcium signals (Lyn–Syk–LAT–SLP-76–Vav1) and this synergy provide competence for MC degranulation. Abbreviations: Ag, antigen; PLCg, phospholipase Cg; PKC, protein kinase C; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase-2; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (Blank and Rivera, 2004).

2.12 SNARE- mediated mast cell exocytosis

The fusion between vesicles or the plasma membrane is not a spontaneous event. They include a conserved molecular machinery of integral membrane proteins- soluble NSF attachment proteins (SNAREs) involved in the fusion of vesicular carriers with the plasma membrane or with other vesicular carriers. The interacting SNARE partners on the opposing membranes assemble through their coiled coil domains to form a parallel four helical bundle structure (Figure 2.11). The so called trans-complex is minimally required for membrane fusion (Puri *et al.,* 2003).

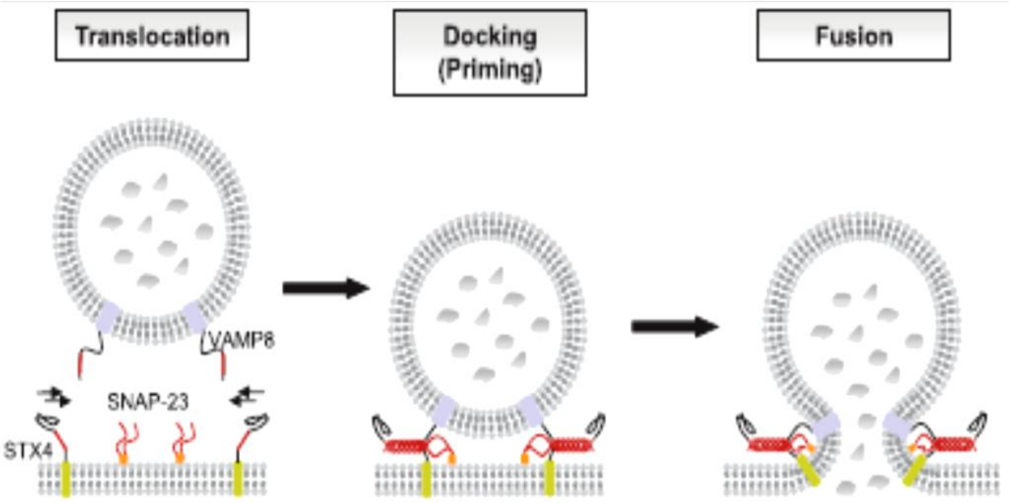

Figure 2.11: SNARE mediated granule fusion in mast cells. The figure shows functional example of SNARE mediated granule fusion in mast cells where secretion of mediators requires fusion of vesicle and plasma membranes. Upon activation through FcεRI secretory granules translocate to and dock at the plasma membrane where the t-SNAREs SNAP-23 and STX4 together with the v-SNARE VAMP8 form stable tetrameric complexes of bundled helices bringing the lipid bilayers into a close distance and catalyze membrane fusion (Lorentz *et al.,* 2012).

Mast cells express a wide array of SNAREs albeit their localization may differ between different cell types and species. To date, described SNARE proteins in mast cells include the t-SNAREs SNAP-23 as well as Syntaxins 2, 3, 4 and 6. VAMP family protein members include VAMP 2, 3, 4, 7 and 8 (Sander *et al.,* 2008; Benhamou and Blank, 2010). Various investigators have reported the expression of multiple SNAREs in the mast cell exocytosis. The RBL-2H3 mast cell line is an extensively studied model for mast cell biology. Immunohistochemistry studies in primary mast cells have demonstrated that SNAP-23 and Syntaxin 4 localize to plasma membrane, while Syntaxin 3, VAMP 2 and VAMP 8 appear to localize to secretotry granules (Guo *et al.,* 1998). A study by Sander *et al.,* 2008 demonstrated that SNAP-23 and Syntaxin 4 localize in the plasma membrane while VAMP 3, VAMP 7 and VAMP 8 are dispersed throughout the cytoplasm, suggesting granule localization. However, upon activation of the mast cell, only VAMP 7 and VAMP 8 appear to redistribute to the periphery of the cell, suggesting fusion and degranulation (Sander *et al.,* 2008).

Immunoprecipitation (IP) pull down studies in the RBL-2H3 cells have identified complexes composed of SNAP-23 and Syntaxin 4 in a complex with the R-SNAREs, VAMP 2, VAMP 8 and VAMP 3 (Paumet *et al.,* 2000). This study also demonstrated that IP with anti SNAP-23 antibody in RBL-2H3 cells pulled down Syntaxin 2, 3, 4 and VAMP 2, 3, 8 while IP with anti Syntaxin 4 resulted in the co-precipitation of SNAP-23, VAMP 2, 3, 8 and IP with anti Syntaxin 2 or Syntaxin 3 only pulled down SNAP-23. These data suggested that the ternary complexes in RBL-2H3 cells consist of SNAP-23, Syntaxin 4 and member of R-SNAREs family, VAMP 2, VAMP 3 or VAMP 8 (Paumet *et al.,* 2000). A study by Pombo *et al.,* 2003 showed that in RBL-2H3 cell lysates, Syntaxin 4 co-precipitated with SNAP-23 and VAMP 8 within and outside lipid rafts. Additional studies demonstrated co-precipitated complexes of SNAP-23, VAMP 2 and Syntaxin 4 in RBL-2H3 cells and most of the SNAP-23 associated with VAMP 2 and Syntaxin 4 in the complexes is phosphorylated (Hepp *et al.,* 2005). Further on, it was demonstrated that SNAP-23, Syntaxin 4 and VAMP 2 complexes are present in lipid rafts and showed that SNAP-23 functions to recruit non-lipid raft associated Syntaxin 4 into a functional complex. It showed that a predominant proportion of the SNAP-23 in complexes was phosphorylated, implicating SNAP-23 phosphorylation as a key prerequisite to complex formation (Puri and Roche, 2006). Using recombinant SNARE proteins, it was demonstrated that SNAP-23 interacts with Syntaxin 4 and VAMP 2, however, the interaction of SNAP-23 and Syntaxin 4 was approximately five times stronger than SNAP-23 with VAMP 2 or Syntaxin 4 with VAMP 2 (Foster *et al.,* 1998). *In vitro* studies of the recombinant SNAREs, showed SNAP 23, VAMP 2 and Syntaxin 4 were associated and further demonstrated that deletion of the amino terminus and the second coiled- coil domain of SNAP-23 inhibited binding to both VAMP 2 and Syntaxin 4 (Vaidhyanathan *et al.,* 2001). All these studies suggest that the important Qb,c and Qa SNAREs on plasma membrane unequivocally appear to be SNAP-23 and STX4, while the most likely R-SNARE partners on the vesicle membranes appear to be both VAMP 7 and VAMP 8. In the absence of VAMP 8, it appears that a compensatory mechanism may allow VAMP 2 and/or VAMP 3 to associate with SNAP-23 and STX4 to mediate ternary complex formation and possible function (Woska and Gillepsie, 2012).

Functional studies implicating the specific SNAREs or SNARE complexes aim to provide the role of these complexes in mast cell degranulation. The most compelling role of SNARE protein in mast cell degranulation is presented in data describing VAMP 8 deficient mice (Puri and Roche, 2008). In some, they possess distinct SNARE proteins (distinct subsets of secretory granules that is based on the requirement of the v-SNARE VAMP 8) regulating the fusion with plasma membrane. VAMP 8 deficient mast cells exhibited defects in FcεRI regulated exocytosis, while synaptobrevin 2 or VAMP 3 defecient mast cells did not show irregulation. The mast cell derived from bone marrow of the VAMP 8 deficient mice, had a 50% decrease in their ability to release β-hexoaminidase, serotonin and cathepsin D but had normal histamine and TNF-α release. The results revealed that mast cells posssess distinct secretory granule subsets, each with different cargo and different functions and indicated that VAMP 8 regulates release of serotonin and cathepsin D from the mast cells (Puri and Roche, 2008). Studies by Tiwari *et al.,* 2009 showed that bone marrow derived mast cells from VAMP 8 deficient mice resulted in 50 % decrease in β- hexoaminidase and histamine but no changes in cytokine/ chemokine release. All these functional findings give a basis of model of SNARE complex mediating degranulation of mast cells.

2.13 Regulation of SNAREs

For the co-ordinate functioning during trafficking and to prevent inappropriate membrane fusion, SNARE proteins have to be regulated. Post-translational modifications of the SNARE proteins and the binding of the regulatory proteins are the two mechanisms that can generate temporal and spatial regulation of trans-SNARE complex formation (Stow *et al.,* 2006). Also, lipid rafts, cholesterol and sphingolipid rich microdomains of the plasma membrane, have been implicated in the regulation of several intracellular pathways. Components of SNARE membrane fusion machinery associate with raft domains, and this interaction may play important role in regulated exocytosis (Salaun and Chamberlain, 2006). The regulatory mechanisms have been studied in immune cells, and mast cells provide the best example. Mast cells are packed with SNARE-decorated granules that must remain inactive in resting cells, but on cell activation, must function rapidly and on a large scale to allow for membrane fusion and degranulation. In case of t-SNARE, SNAP-23 post translational modification can be palmitoylation and phosphorylation. In mast cells, SNAP-23 has potential 7 modification sites, 5 cysteine residual sites (C79, 80, 83, 85, 87) that may undergo palmitoylation modification (Vogel and Roche, 1999). Since, palmitoylation is reversible; it may regulate localization of SNAP-23, thereby regulating membrane fusion. Phosphorylation of SNAREs is also an important regulatory mechanism. In case of SNAP-23, stimulation of the IgE receptor in mast cells cause IKK2 mediated SNAP-23 phosphorylation, which involves regulation of degranulation in mast cells and anaphylaxis (Suzuki and Verma, 2008). Mast cell degranulation involved phosphorylation at Ser95 and Ser120 of SNAP-23 and most of the SNAP-23 was phosphorylated when involved in SNARE complexes with Syntaxin 4 and VAMP 2 after stimulation. Furthermore, over expression of SNAP-23 phosphorylation mutant caused decreased mast cell exocytosis, and phosphorylated SNAP-23 was preferentially associated with Syntaxin 4 and VAMP 2 further suggesting a role for SNAP 23 phosphorylation in regulated mast cell exocytosis (Hepp *et al.,* 2005).

SNARE complex dissociation is mediated by NSF and α-SNAP and these proteins are important regulators of SNARE-mediated membrane fusion. Once membrane fusion is achieved, SNARE proteins remain as a complex in the same membrane (cis-SNARE complex) and have to be reused/ recycled for further rounds of fusion. SNAPs and NSF are cytosolic proteins targeting cis-SNARE complex at all transport steps. NSF is a hexameric ATPase, a member of the AAA protein family. NSF and SNAPs in the cytosol do not interact with each other; NSF only binds SNARE complexes only in the presence of α-SNAP. Three α-SNAP molecules bind to the longitudinal surface of the four-helix SNARE bundle and these molecules recruit N-terminal domain of NSF to bind the SNAP/SNARE complex. Then ATP hydrolysis by NSF dissociates the SNAP-SNARE complex and the four-helix SNARE bundle (Malsam *et al.,* 2008). In the secretory pathway, SNARE disassembly by NSF is crucial for maintaining competence. The use of temperature-sensitive NSF mutants in *Drosophila* has shown that the inactivation of NSF causes the accumulation of SNARE complexes and a block in synaptic transmission (Littleton *et al*., 1998). Transfection of a NSF mutant, was unable to promote SNARE disassembly and markedly inhibited exocytosis, indicating that SNARE disassembly is an integral part of the cycle that primes SNARE mediated fusion (Puri *et al.,* 2003) in mast cells.

Ras-associated binding (Rab) proteins are small GTPases (20-29 kDa) of the Ras superfamily which are ubiquitously expressed and cycle between the cytosol and different membranes (Bock *et al*., 2001). Rab proteins can exist in two different states: a GDP-bound inactive state and a GTP-bound active state. The switch between these two states is controlled by two classes of enzymes: guanine nucleotide exchange factors (GEF, which stimulates the binding of GTP) and GTPase-activating proteins (GAP, which accelerate hydrolysis of the bound GTP to GDP). When Rab is active, it promotes vesicle trafficking through the interaction with specific effector molecules (Fukuda, 2008). Rab effectors bind specifically to the GTP-bound conformation of Rab proteins (Novick and Zerial, 1997). In mast cells, phosphorylation of Q-SNAREs can turn them on or off. In resting mast cells, phosphorylation by Rab3D- associated kinase renders the Qa-SNARE syntaxin 4(STX4) inactive. Whereas, the Qb,c-SNARE SNAP-23 is phosphorylated on cell activation to become functional. (Hepp *et al.,* 2005).

Complexins are cytosolic SNARE regulatory proteins that bind SNARE complexes and lock the SNARE machinery into a primed state awaiting a final trigger of fusion events

(Tadokoro *et al.,* 2005). Specifically, complexin II has been demonstrated to function as a positive regulator of mast cell degranulation (Tuvim *et al.,* 2009). Synaptotagmins are type I membrane calcium binding proteins that facilitate the formation of the SNARE-calcium-phospholipid complex that trigger final fusion and release of mediators. Mostly synaptotagmin II has been shown to be indispensible for mediator release in mast cells (Roa *et al.,* 1997). Further, secretory carrier membrane proteins (SCAMPs), a family of ubiquitous membrane proteins of transport vesicles regulate the fusion. They are highly expressed in organelles undergoing regulated exocytosis as in synaptic vesicles, and also in mast cells (Castle *et al.,* 2002). Mast cells express SCAMP1 to SCAMP3 isoforms, with SCAMP1 and SCAMP2 being most prevalent and they localize to secretory granules and vesicles, while a small fraction is also found at the PM colocalizing with Syntaxin 4 and SNAP-23 (Guo *et al.,* 2002). It acts likely at the final fusion step subsequent to SNAP-23 relocation and ATP dependent priming.

Sec1/Munc18 (SM) proteins play an important role in controlling the process of membrane fusion control. The UNC-18 gene was originally identified through studies performed in *C. elegans* (Brenner, 1974), but homologues have now been described in all organisms examined. For example, a temperature-sensitive mutation in the *SEC1* gene (sec1-1) induced an accumulation of intracellular vesicles demonstrating that this gene is important for vesicle exocytosis in yeast (Novick and Schekman, 1979). SM proteins are hydrophilic proteins of around 60-70 kDa which can be present in the cytosol or attached to membranes *via* their high affinity interaction with syntaxins. Four SM proteins in yeast (*i.e.* Sly1p, Vps45p, Vps33p and Sec1p) and seven SM proteins in mammals (*i.e.* Munc18a, Munc18b, and Munc18c, VPS33A, VPS33B, VPS45, and SLY1) have been identified (Hong, 2005). Munc18a (also termed n-Sec1 or Munc18-1), b (also termed Munc18-2) and c (also termed Munc18-3) are functionally homologous to yeast Sec1p and act at the PM. Munc18a (M18a) was found to be predominantly expressed in the brain (Hata *et al*., 1993), whereas the Munc18b (M18b) and c (M18c) isoforms appeared to be more ubiquitously expressed throughout different tissues (Tellam *et al*., 1995). SM proteins have an essential role in fusion in various types of regulated secretion competent cells, due to their different modes of interaction with cognate SNARE proteins. SM binding to the closed "un-complexed" conformation of syntaxins (an intramolecular interaction of the Habc-domain of syntaxin with its SNARE motif, thereby occluding the SNARE motif), blocks SNARE assembly and could play a role in gating the initiation of the fusion reaction (Blank, 2011). The binding also acts as a chaperone, where the expression of various syntaxins was shown to decrease in the absence of Munc 18 isoforms . In contrast, binding to the trans-SNARE complex may more be directly involved in promoting fusion (Sudhof and Rothman, 2009). Mast cells express the ubiquitous isoforms Munc 18-2 and Munc 18-3. In mast cells, a Syntaxin 3-Munc 18b complex resides in non- lipid-raft membrane domains, and participation of the Q-SNARE Syntaxin 3 in trans-SNARE formation is preceded by movement of Syntaxin 3, without Munc18b into the cholesterol-rich lipid rafts. Hence, both the dissociation of Munc 18b and the cholesterol-rich lipid raft environment operate for regulation of availability and function of Q-SNARE components (Stow *et al.,* 2006).

Lipid rafts are the membrane microdomains and are enriched in cholesterol and glycosphingolipids. They are also known as glycosphingolipid enriched membranes or detergent resistant membranes (DRMs) as they are resistant to solubilization in nonionic detergents at low temperatures. Most of SNARE complexes containing SNAP-23/ Syntaxin 4/ VAMP 3 are found to be associated with lipid rafts in mast cells. Quantitative analysis of multiple experiments showed that 80% of all Syntaxin 4 or VAMP 2 bound to SNAP-23 was present in detergent-insoluble membrane microdomains (Puri and Roche, 2006). Most of SNARE complexes containing SNAP- 23/Syntaxin 4/VAMP 2 are associated with lipid rafts in RBL mast cells. Studies by Puri and Roche, 2006 also showed that Syntaxin 4 association with lipid raft microdomains is a consequence of its binding to the raft-localized SNARE SNAP-23. Lipid rafts have a significant role in the regulation of secretory granules from mast cells.

2.14 Regulatory Domains of SNARE proteins

The neuronal SNARE complex, composed of Syntaxin 1A and VAMP 2 contain one coiled coil domain each, and SNAP-25 contains two coiled coil domains, one at its amino terminus and one at its carboxyl terminus. Deletion analysis has revealed that the neuronal SNAREs are essential for efficient SNARE complex assembly where both the coiled coil domains of SNAP-25 are important for its interaction with VAMP 2, while the first coiled coil is the major Syntaxin binding region (Chapman *et al.,* 1994). The non neuronal homologue of SNAP-25, SNAP-23 is a key player in regulating membrane fusion during exocytosis. The structural domains of SNAP-23 that are essential for interaction with other SNAREs have not been determined fully. In the study by Vaidyanathan *et al.,* 2001 deletion mutagenesis revealed that deletion of the amino-terminal 18 amino acids of SNAP-23(encoded in the first exon) inhibited binding of SNAP-23 to the Syntaxin as well as VAMP. By contrast, truncation of carboxyl-terminal 23 amino acids of SNAP-23 revealed that it was not required for binding of SNAP-23 to syntaxin, while it is essential for SNAP-23 binding to VAMP. For the functional relevance of the modular structure of SNAP-23, wild type human SNAP-23 was over expressed in rat mast cell line and it significantly enhanced exocytosis from mast cells, while it was not observed in transfectants expressing carboxyl terminal VAMP binding mutant of SNAP-23. All the studies demonstrated that SNAP-23 mutants binding to syntaxin but not to VAMP do not support exocytosis from rat mast cells and SNAP-23 C-terminus being important for mast cell exocytosis (Vaidyanathan *et al.,* 2001).

The most studied t-SNARE protein mediating synaptic vesicle fusion, Syntaxin 1 includes a short N-terminal peptide and a larger Habc domain that consists of autonomously folded three helical bundles in the N-terminal regulatory region of the protein (Dulubova *et al.,* 2007). Many *invitro* and *invivo* studies have shown that Muc18-1 binding to N-peptide of Syntaxin 1 is essential for fusion and the Habc-domain of Syntaxin-1 folds back onto the SNARE motif of Syntaxin 1 to produce the closed conformation which also then binds to Munc18-1 (Dulubova *et al.,* 1999). Recent studies by Zhou *et al.,* 2013 showed that N-peptide and the Habc-domain of Syntaxin 1 perform distinct and independent roles in synaptic vesicle fusion. Moreover they showed that deletion of Habc-domain but not the N-peptide deletion caused a loss of binding Munc18-1 protein and it resulted in decrease in readily releasable pool of vesicles at the synapse (Zhou *et al.,* 2013a).

The present predominant model of SNARE mediated fusion suggests that SNARE complex assembly catalyzes membrane fusion by pulling the transmembrane regions (TMRs) of SNARE proteins together, thus allowing to form a fusion pore via TMRs (Sinha *et al.,* 2011). An elegant study by Zhou *et al.,* 2013, demonstrated that lipid anchored Syntaxin 1 and lipid-anchored synaptobrevin 2 lacking TMRs efficiently promoted spontaneous and Ca^{++} triggerd membrane fusion. Replacement of the C-terminal TMR with a lipid anchor in Synatxin 1 and Synaptobrevin 2, did not block the ability of the SNARE proteins to promote fusion, indicating that these SNARE proteins without a TMR still promote fusion (Zhou *et al.,* 2013b).

The complex formed by the mammalian cell surface proteins Munc18c and Syntaxin 4 involves an N-terminal binding mode. Using a range of *invitro* and *invivo* approaches, Latham *et al.,* 2006 showed that the N-terminal 29 amino acids of Syntaxin 4 is critical for its interaction with Munc 18c. They also observed that Munc18c interacts with both monomeric Syntaxin 4 and also with Syntaxin 4/ SNAP-23/ VAMP 2 ternary complexes. The SNARE assembly was increased when Munc18c was allowed to pre-form as a binary complex with Syntaxin 4 (Latham *et al.,* 2006). The data in this paper suggest that Munc18c interacts with its cognate SNARE in a manner that resembles the interaction of yeast proteins Sly1p and Sed5p rather than the mammalian neuronal proteins Munc18a and STX1a. So, even though there is a considerable sequence and structural homology between various SNARE protein family members, it is not possible to predict a common mode of action as different interactions may be observed in case of different SNAREs.

2.15 Molecular Techniques used in the study

Several molecular and biochemical techniques have been used for the study of SNARE proteins. The localization, protein-protein interaction and intracellular trafficking of the SNARE proteins can be examined by expressing them as fluorescent tagged and FLAG tagged fusion proteins in various cells lines. To study the biological functions of a SNARE protein, its expression in the laboratory cell line is the most common approach used. RBL-2H3 cell line has been extensively studied as a model for mast cell biology and various functional studies of SNARE proteins. The expressed proteins are readily detected by western blotting, and are immune-precipitated by antibodies to their relative epitope tags.

In common mode of epitope tagging technique involves fusion of a protein of interest to a peptide epitope that is recognized by a readily available antibody. The epitope tagging can be used for localization of gene products in living cells, identification and characterization of associated proteins (Kozak, 1991). The pCMV- Tag vectors are a series of epitope tagging mammalian expression vectors which are derived from the pCMV-Script vector and contain sequences for either the FLAG or c-myc epitope at either N or C terminus. The specific epitope tags are small, highly immune-reactive and not readily interfere with the function of target protein. In addition, pCMV-Tag vectors, for expression of fusion proteins in eukaryotic cells, have the cytomegalo virus (CMV) promoter. It allows constitutive expression of the cloned DNA in a variety of mammalian cell lines. The neomycin-resistance gene is under control of both the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells. The multiple cloning site (MCS) of the pCMV-Tag vectors allows for a variety of cloning strategies, resulting in either C-terminal or N-terminal fusions with either FLAG or c-myc. A Kozak consensus sequence provides optimal expression of the fusion protein when the N-terminal FLAG epitope is used (Kozak, 1991).

Sub-cloning of mammalian genes into a mammalian promoter-driven expression vector and expressing these genes in the cell line has been used for multiple functional studies of various SNAREs. Expressing and tagging the proteins can be done in expression vectors, as these contain various epitope tags and multiple cloning sites. An expression vector has features of any vector like origin of replication, a selectable marker, and a suitable site for the insertion of a gene such as the multiple cloning sites. The protein coding regions of a gene, without non-coding or non- interesting regions can be transferred.

The molecular machines that are protein complexes assembled and dismantled in particular locations within the cell and the workings of the proteins in living cells are well recognized now by the use of fluorescent tags that track the individual protein in the cell. Fluorescence methods are now commonplace and the fluorescent molecule are effectively self luminous. The simplest application of fluorescent tagging of protein is to attach a fluorophore to a protein to mark where it goes or expressed (Whitaker, 2000). Immunofluorescence uses the recognition of cellular targets by fluorescent dyes or antigen specific antibodies coupled to fluorophores. Confocal microscopy relies on the combination of point illumination and a pinhole to eliminate most of the out-of-focus light signal and allows for reconstruction of 3D volumes, making it ideal to image cells.

The use of green fluorescent protein (GFP) as a reporter molecule is widely used and requires only oxygen and illumination by proper wavelength of light, without any exogenous substrates, co-factors or antibodies for detection, thereby facilitating non-invasive, *in vivo* detection (Prasher *et al.,* 1995). GFP retains fluorescence when fused to another protein at either the N-terminal or C-terminal and this property of GFP makes it ideal fluorescent tag to monitor sub cellular localization of proteins, organelles and biochemical processes. GFP serves as unique reporter and used as a fusion tag for monitoring protein localization or to visualize responses of cells and even tissues or whole organisms to a variety of biological events or signals. GFP is used as a tag in a fusion protein where it is coupled to the protein whose expression is to be tracked. In such cases, the principle aim is to investigate the sub cellular localization of the protein under investigation. Genetic engineering can be used to produce vectors containing a GFP coding sequence into which a coding sequence for a protein can be cloned. The resulting GFP-tagged fusion construct can be transfected into suitable target cells and expression of the GFP-tagged fusion can be monitored to track the subcellular location of the protein (Takuma *et al.,* 2002). The red fluorescent protein cloned from *Discosoma spp.* coral (DsRed or drFP583) holds great promise for biotechnology and cell biology as a spectrally distinct companion or substitute for GFP.

Flow cytometry is a technique that uses the principles of light scattering, light excitation, and fluorescence to analyze size, complexity, molecular or cellular characteristics of single cells or particles by suspending them in a stream of fluid. This technique is widely used to analyze the expression of both extracellular and intracellular proteins, secreted molecules and DNA content, among others. Among the main advantages of flow cytometry are the multi-parametric analysis of mixed populations by identifying specific markers on these cells and (Cunningham, 2010) the separation of particular subpopulations by electrical or mechanical means to divert cells with measured characteristics within the range specified by the user (Krutzik *et al*., 2004). Utilization of multi color flow cytometry allows for measurements of multiple proteins in parallel, regardless of the protein length (Krutzik *et al*., 2004). Intracellular flow cytometry, in comparison with conventional cell surface labeling methods, requires fixation and permeabilization of the cells before staining of intracellular antigens.

MATERIAL AND METHODS

3.1 Plasmids and vectors used in the study

Rat Syntaxin 4(wild type and mutant) cloned in pCMV-Tag 2B vector (Figure 3.1) was a kind gift from Dr. Paul A. Roche (National Institute of Health, Bethesda, MD, USA). The plasmids used in the study are referred to as pCMV-STX4$_{WT}$ and pCMV- STX4$_{mut}$.

3.2 Cloning and expression system

The vector pEGFP-C2 vector (Clontech Laboratories, Inc. Genebank Accession # U57606); (Figure 3.2) vector containing EGFP gene, including neomycin resistant gene were used in the experiments. pEGFP-C2 has been optimized for brighter fluorescence and higher expression in mammalian cells. Any gene cloned into the MCS will be expressed as fusion to the C terminus of EGFP if they are in same reading frame as EGFP and there are no intervening stop codons. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method and can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker).

Also, the expression vector, pDsRed1-C1 (Clontech Laboratories, Inc) (Figure 3.3), a novel red fluorescent protein that is optimized for high expression in mammalian cells was used in the study. pDsRed1-C1 can be used to construct fusions to the C-terminus of DsRed1 and a fusion construct retaining the fluorescent properties of the native DsRed1 protein, its expression and localization *invivo* is monitored by fluorescence microscopy of flow cytometry. The vector can be simply used to express DsRed1 in a cell line of study as a co-transfection marker.

3.3 Enzymes, chemicals and reagents

Molecular biology reagents- Chloroform, Isopropanol, Ethanol, EDTA, Trypsin, Tris-base, Boric acid, Agarose were from SIGMA. Media component (LB) was purchased from Himedia and other reagents were obtained from commercial suppliers and were of analytical grade. DNA ligase was from NEB (Quick Ligase). DNA markers were from NEB and Fermentas. Restriction enzymes used in the study (Chart shown below) were obtained from New England Biolabs (NEB) Inc. UK.

Enzyme	**Recognition site**	**Supplied NEBuffer (10X)**
*Apa*I	GGGCCC	NEBuffer 4+ BSA
*Bam*HI	GGATCC	NEBuffer 3+ BSA
*Eco*RI	GAATTC	NEBuffer EcoRI
*Hind*III	AAGCTT	NEBuffer 2
*Xho*I	CTCGAG	NEBuffer 4+ BSA

3.4 For cell culture

TIB142 culture (IgE secreting hybridoma) was obtained from ATCC and its supernatant was generated at Cellular and Molecular Immunology Laboratory, SLS, JNU. DNP-BSA, RPMI 1640 Media, Minimal Essential Medium (MEM) and Iscove’s media, Trypsin, Phosphate Buffered Saline (PBS), Sheath Fluid, Fetal Bovine Serum (FBS), Trypan Blue , Triton X-100, Sodium Citrate. The stimulating agents were dissolved in RPMI 1640 (without L-glutamine, without phenol red) and all were obtained from SIGMA and hGH kit from (Roche diagnostics).

3.5 Maintenance of Cell line

The Rat Basophilic Leukemia (RBL-2H3) mast cell line was a kind gift from Dr. Paul A Roche (NIH, USA). They were maintained in equal parts of Minimal Essential Medium and Iscove’s Modified Dulbecco’s Medium containing 20% Fetal Bovine Serum (FBS) (Gibco), 25mM HEPES, and 120 µg/ml gentamycin, referred to as RBL complete medium. Cells were maintained as subconfluent monolayers at 37°C in a humidified atmosphere containing 5% CO_2 and passaged with trypsin.

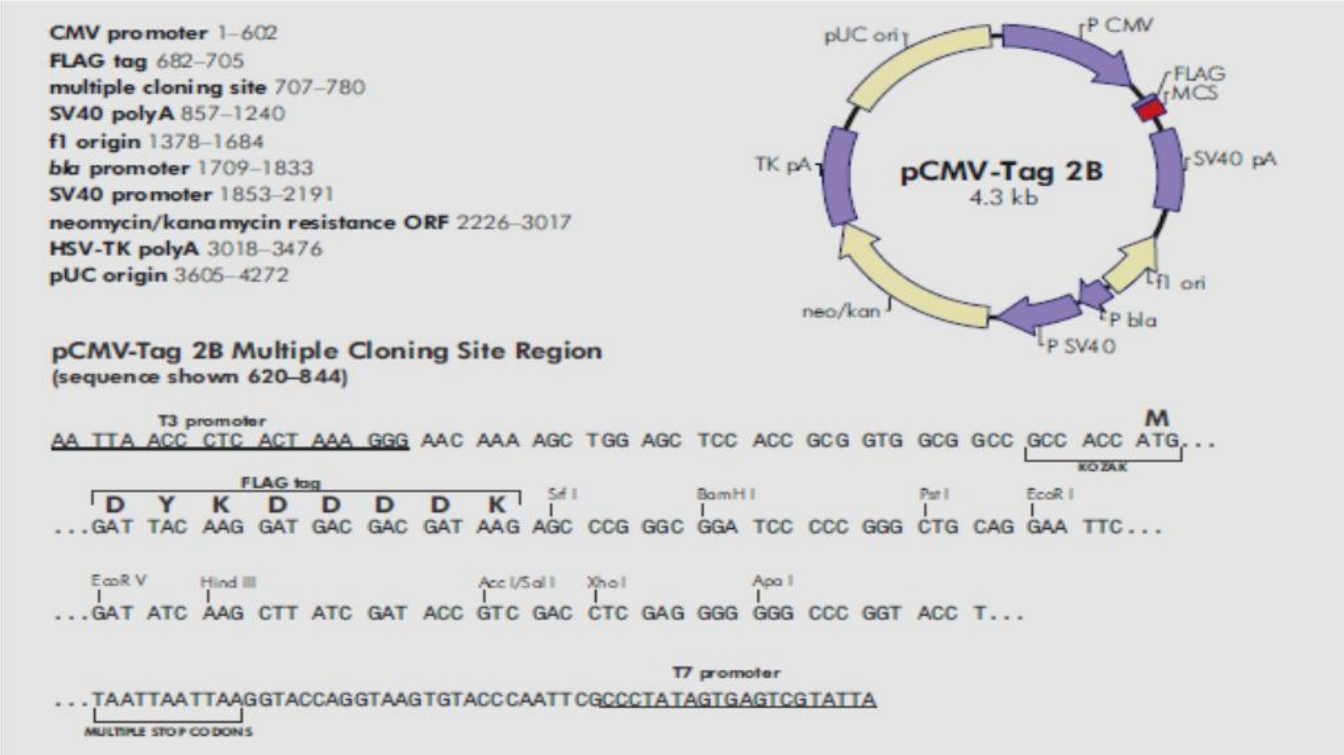

Figure 3.1: Restriction map and Multiple Cloning Site (MCS) of pCMV-Tag 2B vector

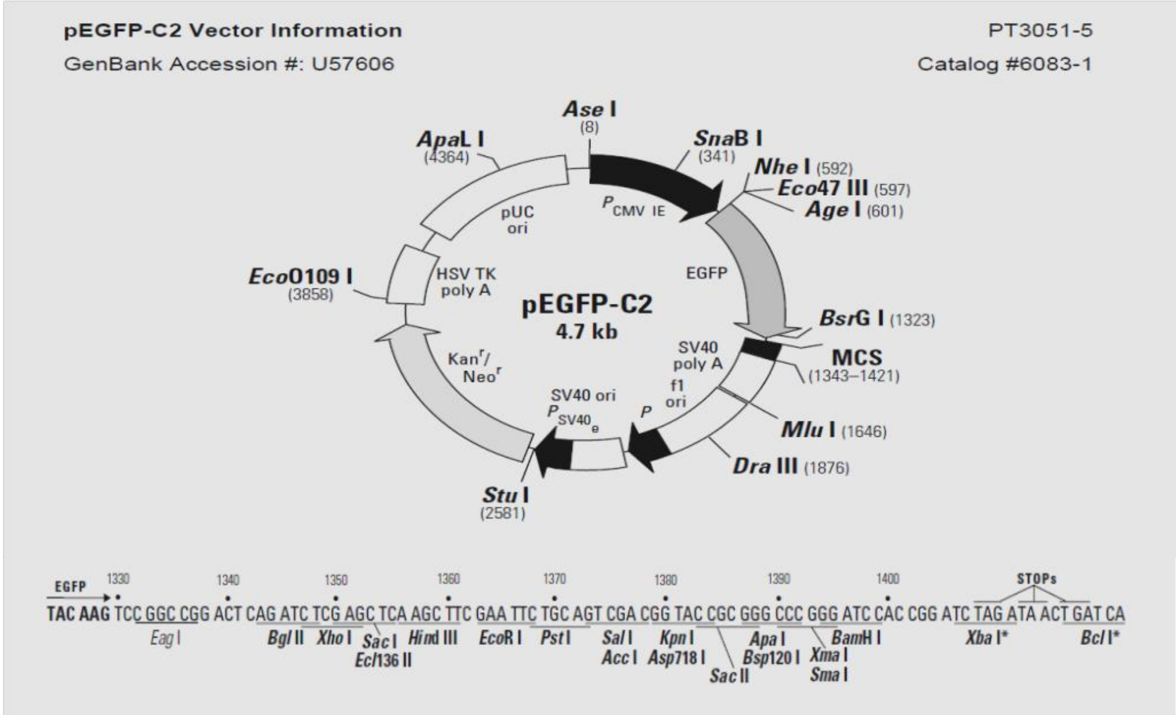

Figure 3.2: Restriction map and MCS of pEGFP-C2 vector

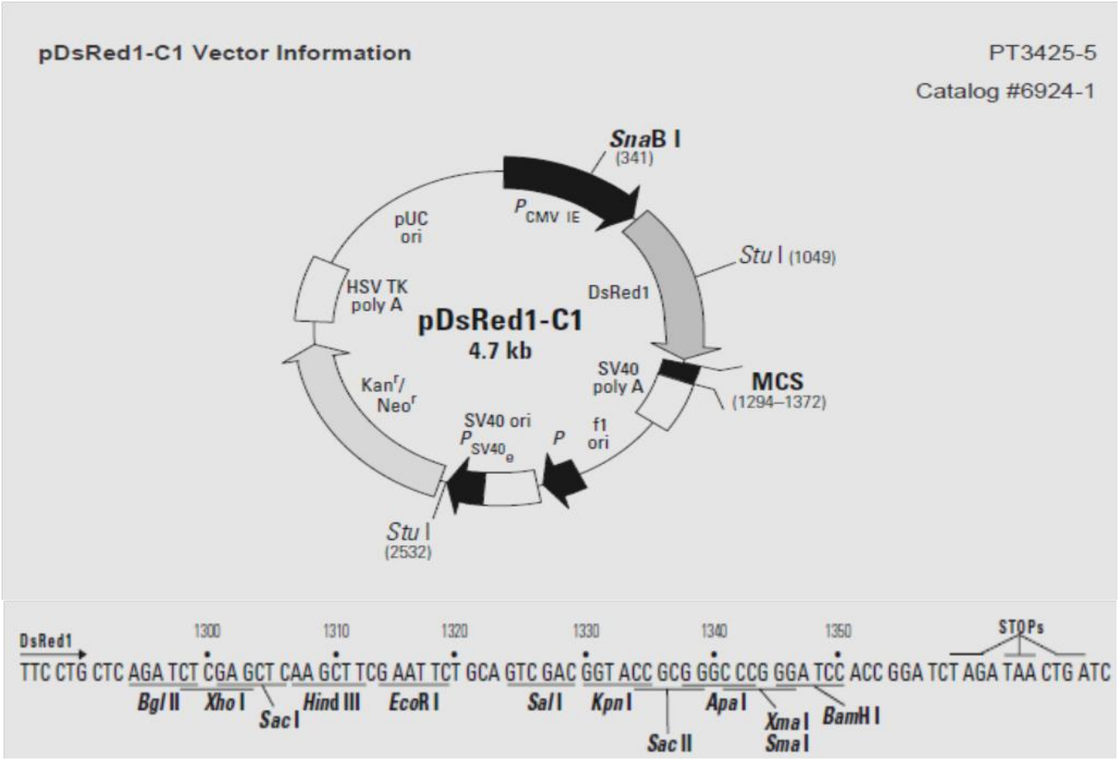

Figure 3.3: Restriction map and MCS of pDsRed1-C1vector

3.6 Preparation of Competent Cells

The competent *E. coli* cells were prepared as described by Sambrook and Russel, 2001. Briefly, *E. coli* DH5α cells (Invitrogen) from frozen glycerol stock or from earlier competent cells' vial were streaked on LB plates and incubated overnight at 37^{o}C. A single colony was picked and inoculated in 3 ml of LB medium and incubated at 37^{o}C for overnight kept in shaker/incubator. 200 µl of overnight grown cells was used as inoculums to obtain culture at log phase by inoculating in 50 ml LB media in a flask. The flask was incubated at 37^{o}C till the optical density at 600 nm reached 0.5-0.6, taking LB broth as control. Cells were kept on ice for 5-10 min. Then cells were collected by centrifugation at 6000 rpm at 4^{o}C for 10 min. Supernatant was drained off completely and the obtained cell pellet was resuspended in 24 ml of freshly prepared 0.1M $MgCl_2$ and 6 ml of 0.1 M $CaCl_2$. The pellet was dissolved properly by gentle pipetting and incubated on ice for 5 min. The suspension was centrifuged at 6000 rpm at 4^{o}C for 10 min and the obtained pellet was resuspended in 1.5 ml of ice cold 0.1 M $CaCl_2$. Tubes were sealed with parafilm and kept overnight at 4^{o}C. The competent cells were stored as 10% glycerol stocks in small aliquots in eppendorf tubes and stored at -80^{o}C. The competence efficiency of the prepared competent cells was then checked by transformation.

3.7 Transformation of Competent *E. coli* with Plasmid Construct

Chemically competent cells (*E. coli* DH5α), usually containing 75 µl/tube were stored at -80°C until use. The stored competent cells were thawed keeping on ice for 10 minutes. Briefly, 10-100 ng of DNA (pCMV-STX4$_{\mathbf{WT}}$ or pCMV-STX4$_{\mathbf{mut}}$) was added to 75 µl competent cells in a 1.5 ml eppendorf tube, gently mixed by pipetting and further incubated for 15 min on ice. Cells were briefly heat shocked at 42°C for 60 sec in water bath and then immediately chilled on ice for 2 min. Tubes were removed from ice and to the mixture, 925 µl SOC (Super Optimal broth with Catabolite repression) medium was added and cells were allowed to grow at 37°C for 1 hr in a shaker incubator at 225 rpm. The *E. coli* cultures were centrifuged at 10,000 rpm for 5 min and 925 µl of the supernatant was removed. Then pellet was resuspended in the remaining 75 µl SOC medium and plated onto the kanamycin (50 µg/ml of media) containing LB agar plate and incubated overnight at 37°C. Small colonies of *E. coli* that were able to grow on media selected with kanamycin after incubation would have been transformed by the plasmid used pCMV-STX4$_{\mathbf{WT}}$ or pCMV-STX4$_{\mathbf{mut}}$.

The efficiency of the transformation was calculated by using the following formula:

$$\text{Transformation efficiency(transformants µg)} = \frac{\text{Total number of colonies formed}}{\text{Amount of plasmid DNA added (in µg)}} \text{x } 1000$$

Usually for good chemically competent cells, 10^5 to 10^6 cfu/ µg DNA are obtained.

3.8 Mini preparation (Small scale) plasmid DNA preparation

3.8.1 Harvesting of Bacterial Cells

A single bacterial colony of *E. coli* (DH5α) cells containing the desired plasmid was picked up and inoculated into 5 ml LB media containing appropriate antibiotic (50 µg/ml kanamycin) in a loosely capped 15 ml falcon tube and grown overnight at 37°C with shaking at 225 rpm. 1.5 ml of culture was poured into an eppendorf tube and centrifuged at 12000 rpm for 30 sec.

3.8.2 Alkaline Lysis

The cell pellet was resuspended in 100 µl of ice-cold Solution I by vigorous vortexing and to it 0.5 µl of RNase (Fermentas) was added and mixed by tapping. The tube was incubated on ice for 5 min followed by addition of 200 µl of freshly prepared Solution II. The tube was inverted 5-10 times to mix the contents and incubated on ice for 3 min.

then, 150 µl of ice-cold Solution III was added, mixed by inversion of tube for several times and incubated on ice for 5 min. The contents were then centrifuged at 12000 rpm for 10 min at 4^{o}C to remove cell debries. The supernatant was collected in a fresh tube carefully and to it equal volume of phenol:chloroform was added and vortexed gently. The suspension was centrifuged at 13000 rpm for 2 min and 30 seconds and the upper aqueous phase was collected in a fresh tube and to it 0.6 volume of isopropanol was added. The contents were gently mixed by inversion of tube and incubated on -20^{o}C for 1 hour. The tube was then centrifuged at 12000 rpm for 5 min and the supernatant was drained off. To the obtained pellet, 50 µl of 70% ethanol was added, pellet rinsed by gently rotating the tube and centrifuged at 12000 rpm for 5 min. the alcohol was decant off completely and carefully and the DNA pellet was air dried till the last drop of alcohol dried off. Finally, plasmid DNA was dissolved in 50 µl 10mM Tris (pH-8) and stored at -20^{o}C.

3.9 Maxi preparation (Maxiprep) of Plasmid DNA (by Alkaline Lysis method)

3.9.1 Harvesting of Bacterial Cells

A single colony of *E. coli* (DH5α) cells containing the desired plasmid construct was picked up and inoculated into 5ml LB media containing appropriate antibiotic (kanamycin 50 µg/ ml of media) in a 15 ml falcon tube and grown overnight at 37^{o}C with shaking at 225 rpm. 2.5 ml of this log phase primary culture was transferred into 500 ml LB media containing antibiotic in a 2 liter conical flask and grown for 16-18 hours at 37^{o}C with shaking at 250 rpm. The cells were then harvested by centrifugation at 6000 rpm for 15 min at 4°C in Hitachi R_{10} A3 rotor. The supernatant was drained off and the pellet was resuspended in 50 ml of ice cold STE solution. The solution was centrifuged at 6000 rpm for 15 min at 4°C.

3.9.2 Alkaline Lysis

The cell pellet was resuspended in 18 ml of ice cold Solution I with vigorous shaking and then 20 µl of RNAse (10 mg/ml) was added. Thereafter, 25 ml of freshly prepared Solution II was added and mixed gently by inverting several times and then incubated at room temperature for 10 min. To the cell suspension 15 ml of ice cold Solution III was added and mixed thoroughly by gentle inversion of the tubes for many times the tubes and then incubated on ice for 10 min. The whole content was centrifuged at 9000 rpm for 40 min at 4°C to recover bacterial lysate and the supernatant was filtered through two layers of cheese cloth. To the filtrate obtained, 0.6 volume of isopropanol was added, mixed by inversion and then incubated at room temperature for 10 minutes. Tubes were then centrifuged at 9000 rpm for 20 min at room temperature. The pellet was washed with 70% ethanol and then centrifuged at 9000 rpm for 5 min. The DNA pellet was allowed to air dry and then resuspended in 1.5ml of 10mM Tris (pH-8) at 37^{o}C and finally stored at -20^{o}C.

3.10 Purification of plasmid DNA after Maxiprep (by Phenol: Chloroform extraction)

To the DNA suspension prepared equal volume of equilibrated phenol: chloroform (1:1) solution was added, vortexed gently and mixed by inversion of tube. The contents were centrifuged at 13000 rpm for 10 min 4°C and the aqueous phase was collected in a fresh tube. To it equal volume of chloroform was added, vortexed gently and mixed by inversion. The tube was then centrifuged at 13000 rpm for 10 min at 4°C. To the aqueous phase obtained, 25 µl of Solution III and 0.6 volume of isopropanol was added, incubated at -20°C for 1 hour and then centrifuged at 13000 rpm for 20min at 4°C. The pellet obtained was rinsed with 70% alcohol by gentle rotation of tube and centrifuged at 13000 rpm for 5 min at 4^{o}C. Finally the DNA pellet was air dried and dissolved in 1ml of 10mM Tris (pH-8) and stored at -20^{o}C.

3.11 Determination of Concentration and Purity of DNA using Nanodrop ND2000 Spectrophotometer

DNA samples isolated by both mini preparation and maxi preparation were quantified by using Nanodrop ND2000 Spectrophotometer. The purity of the DNA sample was calculated by taking a ratio between the readings at 260 nm and 280 nm.

DNA purity ratio = A260/A280

Where, A260 and A280 are Optical density (OD) of DNA sample determined at wavelengths 260 nm and 280 nm respectively.

Pure DNA preparation has A260/A280 values equal to 1.8, preparation of DNA having RNA as impurity has A260/A280 ratio greater than 1.8 while samples having protein contamination have A260/A280 values less than 1.8. The absorbance at 260 nm provides an estimate of the concentration of the nucleic acid in the sample.

Concentration of Plasmid DNA = 50 x A260 nm x dilution factor

Where, A260 = optical density of Plasmid DNA determined at 260 nm. An OD260 of 1 corresponds to approximately 50 µg/ml of DNA.

3.12 Restriction Digestion of Plasmid DNA

Enzymes either single or pair of enzymes were chosen depending on the restriction sites of interest. The buffer used in the reaction was selected according to its compatibility with the enzymes. The restriction digestion mixture was set up as:

Restriction digestion mixture (Single Digestion)

Autoclaved MiliQ	16.5 µl
10X *Eco*RI Buffer	2 µl
DNA (0.5 – 1 µg)	1 µl
Enzyme (*eg: Eco*RI)	0.5 µl
Total	20 µl

Restriction digestion mixture (Double Digestion):

Autoclaved MiliQ	16 µl
NEB buffer 4(10X)	2 µl
BSA	0.2µl
DNA (0.5- 1 µg)	1 µl
Enzyme 1 (*Eco*RI)	0.5 µl
Enzyme 2 (*Apa*I)	0.3 µl
Total	20 µl

Samples were mixed and tubes were spun briefly prior to incubation at 37^{o}C for 3-4 hrs. After digestion was completed, those products were analyzed by agarose gel electrophoresis.

3.13 Agarose gel electrophoresis for digested plasmid DNA

Agarose gels were usually made containing 0.6 % agarose in 0.5X TBE (0.6 gm of agarose powder in 100 ml of TBE buffer), placed in a 250ml glass bottle and whole solution was melted in microwave oven until all agarose completely dissolved. When the solution was cooled down to 50-60^{o}C, 1.5 µl (0.15 µg/ml) of ethidium bromide was added to it. Following quick mix, gel cassette was prepared and allowed to solidify after which the gel was transferred into a DNA gel tank containing 0.5X TBE. DNA samples were loaded as:

- 3.3 µl 6X loading dye+ 20 µl Restiction digested sample → Loaded 10 µl in each well
- 1 µl undigested DNA+ 5 µl Autoclaved MiliQ+ 1 µl 6X loading dye → Loaded 6 µl in a well
- 1 µl 1 kb NEB marker+ 5 µl Autoclaved MiliQ+ 1 µl 6X loading dye → Loaded 6 µl in a well

The gel electrophoresis was performed at 80 volts for 3 hours and the gel was observed under Biorad GELDOC System. The plasmid DNA was also quantified using BIORAD Quantity One software.

3.14 Restriction digestion of plasmids and vectors

In all cloning experiments, the vectors pEGFP-C2 and pDsRed1-C1 were cut by double digestion by the appropriate restriction enzymes *Eco*RI and *Apa*I. The DNA fragments to be cloned $STX4_{WT}$ and $STX4_{mut}$ were produced by restriction digestion of the source vector by same set of enzymes. The restriction digestion mixture was set up as:

Autoclaved MiliQ	32.5 µl	Autoclaved MiliQ	37.5 µl
NEB buffer 4(10X)	5µl	NEB buffer 4 (10X)	5 µl
BSA	0.5µl	BSA	0.5 µl
Insert	10 µl	Vector	5 µl
Enzyme 1 (*Eco*RI)	1 µl	Enzyme 1 (*Eco*RI)	1 µl
Enzyme 2 (*Apa*I)	1 µl	Enzyme 1 (*Apa*I)	1 µl
Total	50µl	Total	50µl

The digested samples were mixed and tubes were spun briefly and then incubated at 37°C for 2-3 hrs. To eliminate the background recirculisation, vectors were dephosphorylated by incubating with Shrimp Alkaline Phosphatase (SAP) (Fermentas) (1 µl/µg DNA) for 1 hr at 37°C. Then the digested insert and SAP treated vector DNA were heat inactivated at 65°C for 20 minutes and then run on the gel.

3.15 Gel Electrophoresis and DNA extraction from gel

Firstly the samples were run along with marker on 0.6% agarose gel. Inorder to extract the DNA band of interest, agarose gels were firstly examined under UV light in a spectrophotometer and a clean scalpel was used to excise the correct DNA band. The excised gel band was the transferred into a sterile, pre-weighed 2 ml microcentrifuge tube and the DNA was purified using the QIAGEN (QIAquick) gel extraction kit.

The QIAquick Gel Extraction kit protocol is designed to extract and purify DNA of 70bp to 10kb from standard low-melt agarose gels in TAE or TBE buffer. The manufacturer's instructions were carefully followed. Briefly, the weight of gel was measured and 3 volumes of Buffer QG was added to 1 volume of gel (for example for 100 mg of gel, 300 µl of Buffer QG added). The gel slice was melted at 50°C for 10 min until the gel slice completely dissolved. Then, 1 gel volume of isopropanol was added to the sample and mixed well. The solution was then placed into a QIAquick spin column (800 µl of solution at a time) and centrifuged at 13000 rpm for 1 min at room temperature. The flow through was discarded and the QIAquick column was kept back into the same collection tube. 500 µl of Buffer QG was added to the column for complete removal of all traces of agarose and centrifuged at 13000 rpm for 1 min. Then 750 µl of Buffer PE (containing ethanol) was added to column in order to wash it and centrifuged at 13000rpm for 1 min. The flow through was discarded and to make sure all ethanol was removed, the column was centrifuged for an extra 1 min. Finally, the column was placed into a 1.5 ml microcentrifuge tube and 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the centre of the column and allowed to incubate for 1 min at room temperature. The column was centrifuged at 13000 rpm for 1 min to collect the DNA and stored at -20°C.

3.16 Quantification of vector/insert and DNA ligation

The concentration of the gel purified DNA was determined by gel quantification against the known amount of standard DNA ladder. The vector and insert were then mixed at a ratio of 1:5 respectively using formula given below and ligated using a standard ligation protocol.

$$\text{ng of insert} = \frac{\text{ng of vector to be added x kb size of insert}}{\text{kb size of vector}}\ x\ \frac{\text{Molar ratio of insert}}{\text{Molar ratio of vector}}$$

The complementary overhangs of the vector and the insert were ligated under the action of T4 DNA ligase enzyme (NEB).

The ligation mixture was setup as:

Vector (50ng)	5 µ	Vector (50ng)	5 µl
Insert (STX4$_{\mathbf{WT}}$) (47.71ng)	8 µl	Insert (STX4$_{\mathrm{mut}}$) (19.68ng)	8 µl
Nuclease free water	4 µl	Nuclease free water	4 µl
10X T4 ligation buffer	2 µl	10X T4 ligation buffer	2 µl
T4 DNA ligase	1 µl	T4 DNA ligase	1 µl
Total	20 µl	Total	20 µl

Samples were mixed and tubes were spun briefly and then incubated at room temperature for 10-20 min. Immediately after ligation, half of the ligation mixture was used to transform *E. coli* DH5α competent cells as the method described previously.

3.17 Confirmation of Transformants

3.17.1 Plasmid DNA Extraction

The isolated single kanamycin resistant transformed colonies were picked from agar plate, inoculated into antibiotic containing LB medium, incubated overnight at 37$^{\mathrm{o}}$ C with shaking and then plasmid DNA was isolated by Alkaline Lysis method as described previously.

3.17.2 Restriction Digestion

The recovered plasmid DNA was double digested with same restriction enzymes to verify the cloning. Typical 20 µl digestion setup was prepared, incubated at 37$^{\mathrm{o}}$ C for 3-4 hrs and electrophoresed in 0.6% agarose. The fall out of desired band observed on gel confirmed the positive transformants with the desired DNA construct cloned in the vector. After that the positive DNA constructs were analyzed by DNA sequencing in order to ensure that all sequences were correct.

3.18 Cell culture and Cell Counting using Haemocytometer

RBL-2H3 cells were grown in medium containing equal parts of Minimal Essential Medium and Iscove's medium supplemented with FBS, HEPES and gentamycin (described earlier). Exponentially growing cells were maintained as adherent culture in the RBL complete medium at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured by trypsinization.

Initially, 0.2×10^5 RBL cells per ml RBL media were seeded in 24 well tissue culture plates. Cells were harvested by trypsinzation and counted on haemocytometer using Trypan blue staining.

Haemocytometer is a thickened glass slide having a small chamber of grids cut into the glass. The counting chamber is etched in 9 large squares each measuring 1mm x 1mm in area and 0.1mm in depth (Figure 3.4). Each one mm square is divided into 25 medium sized squares (groups) (0.2 mm x 0.2 mm each), each of which is further subdivided into 16 small squares (0.05 x 0.05mm each), thus a total of 400 squares in 1 mm. Each large square has a volume of 1x1x0.1 mm= 10^{-4} cm^3.

Cell count can be estimated using the formula:

Number of cells counted in total volume of sample = Total number of cells counted x total volume of sample x Dilution factor x 10^4

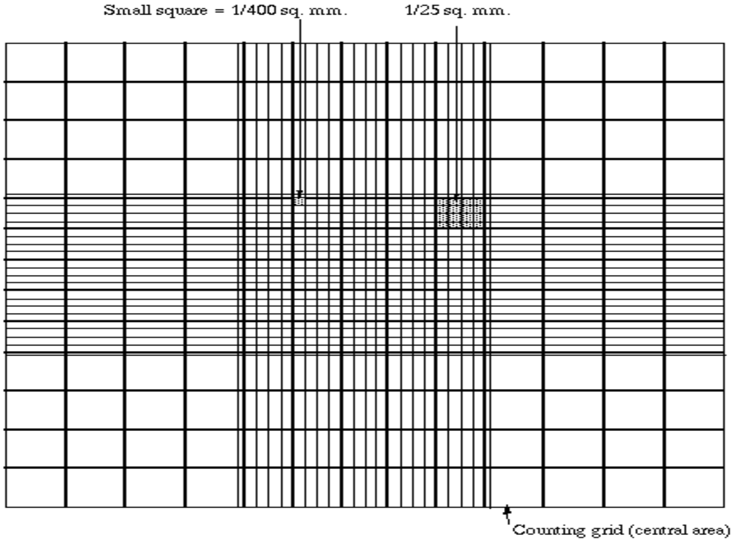

Figure 3.4: A haemocytometer grid

Trypan Blue is called a vital dye as it is negatively charged and does not interact with the cell unless the membrane is damaged (Freshney RI, 2010). Therefore, all the cells which exclude the dye appear as bright translucent structures and are viable whereas the dead cells stain blue. For cell counting, the sample (10µl of cell suspension) was taken and 90µl of 0.2% trypan blue was added to it.10µl of the suspension was loaded on hemocytometer and cells were counted under the microscope.

3.19 Transfection of RBL-2H3 cells

3.19.1 DNA preparation

In a sterile eppendrof, 20 µg DNA, 180 µl autoclaved MiliQ, 0.5 µl glycogen, 20 µl Alkaline Solution III and 500 µl chilled ethanol were mixed by inversion and incubated overnight at -20^{o}C (Puri *et al.,* 2003). The whole mixture was then centrifuged at 13000 rpm for 30 min at 4^{o}C. The pellet obtained was washed with 50 µl of 70% alcohol and centrifuged at 13000 rpm for 8 min at 4^{o}C.The DNA pellet was dried properly and then dissolved in 25 µl of Mol. Bio grade water. The mixture was kept at 37^{o}C and immediately used for transfection.

3.19.2 Transfection

Exponentially growing RBL cells were harvested by trypsinization, and then cells were washed twice with antibiotic free RBL medium and once with serum free and antioboitic free RBL medium. Cells were counted on haemocytometer and then re-suspended in serum-free and antibiotic-free RBL medium (10 x 10^{6} cells /0.5ml medium). The cells, DNA and transfection cuvettes (0.4 cm) along with 10 ml antibiotic free RBL media containing 10 cm culture dish, were prewarmed at 37^{o}C. In the electroporation cuvette, 500 µl cell suspension+ 25 µl DNA (per transfection) were mixed by tapping and then electroporated at 320V and 950µF in Bio-Rad Gene Pulser (as described in Puri *et al.,* 2003). Transfected cells were immediately plated in pre warmed antibiotic-free RBL medium in tissue-culture dishes and analyzed after 24-48 hours by either flow-cytometry or fluorescence microscopy.

3.19.3 Flowcytometry

24-48 hours post-transfection cells were harvested by trypsinization. Cells were washed twice with antibiotic-free RBL medium and once with PBS containing 10mg/ml BSA. Cells were re-suspended in PBS and analyzed by BD FACS Calibur flow cytometer using Cell Quest software. 10,000 cells were acquired in each experiment. Live cells were gated on the basis of their FSC and SSC. Positive transfected cells were gated on the basis of their fluorescence intensity in green (FL1) channel.

3.19.4 Fluorescence Microscopy

RBL cells transfected with fluorescent tagged EGFP were seeded RBL cells were grown on coverslips. After 12-24hrs, cells were fixed with 4% Paraformaldehydae (PFA) for 20-30 min at RT followed by washing with quencher for two times. Excess paraformaldehyde was quenched with 50mM NH_4Cl in PBS. Coverslips were mounted in Fluoromount G and microscopic images were collected with Nikon Eclipse Ti microscope at 60X magnification.

3.20 RBL exocytosis assay (Induction of Secretion in RBL cells by IgE crosslinking)

RBL cells transfected with 2 µg of plasmid encoding hGH together with the test plasmid were grown in 24-well plate (0.3x 10^6 cells/1.5 ml RBL complete media/ well), overnight so as to become confluent (as described in Puri *et al.,* 2003). Media was aspirated next day from the wells followed by a wash with phenol red-free RPMI (PR^-). Degranulation was triggered with respective stimulants of varying concentrations and incubation periods. For stimulation by IgE cross-linking, subconfluent RBL cells were sensitized with DNP-specific IgE (TIB 142 tissue culture supernatant at 1:100 dilutions) in RBL complete medium and incubated overnight at 37°C. After 16-18 hours, media was removed, gently washed along the wells with phenol red negative RPMI for two times. IgE sensitized cells were stimulated with 100ng/ml DNP-BSA for 15-45 min in CO_2 incubator (Vaidyanathan *et al.,* 2001). Cell supernatant was collected, cells washed twice with ice cold phenol red-free RPMI (500 µl per well) and then cells lysed in phenol red-free RPMI containing 0.2% Triton X-100 by 1 hour shaking on gel rocker, with plate kept on ice. Then the lysates were collected and stored at -80°C. The amount of hGH released into the culture supernatant and remaining in the cell lysates was determined using human growth hormone enzyme-linked immunosorbent assay (hGH ELISA) kit from Roche Diagnostics, as per manufacturer's instructions and expressed as a proportion of the amount of hGH released in the supernatant and total hGH in the cells (Puri *et al.,* 2003).

The hGH ELISA is based on the sandwich ELISA principle. Antibodies to hGH (anti-hGH) are prebound to the surface of the microplae modules and following transfection of cells, the cell supernatant, which contains secreted hGH is added to the wells of MP modules. All hGH contained in the medium binds specifically to the anti-hGH antibodies bound to the microplate surface. Next, a digoxigenin-labelled antibody to hGH (anti-hGH-DIG) is added and binds to hGH. In the following step, an antibody to digoxigenin conjugated to peroxidase is added and binds to digoxigenin. In the final step, the peroxidase substrate ABTS is added and it catalyzes the cleavage of the substrate yielding a colored reaction product. The absorbance of the sample is determined using microplate (ELISA) reader at 405 nm with a reference wavelength at 490 nm and the readings directly correlate to the level of hGH present in the medium supernatant.

RESULTS

4.1.1 Devising a strategy to clone rat STX4$_{WT}$ and truncated rat STX4$_{mut}$ into pEGFP-C2 and pDsRed1-C1 plasmid vectors

The plasmids used in the study- rat STX4$_{WT}$ (full length) and STX4$_{mut}$ (truncated) cloned into pCMV-Tag-2B FLAG vector were obtained as kind gifts from Dr. Paul A Roche (NIH, USA). In order to confirm the sequence of cloned constructs and to determine the restriction enzyme sites available for recloning, two strategies were used. First, the plasmids were amplified by Maxiprep and restriction digestion was performed by different sets of enzymes chosen from the MCS of the original pCMV-Tag-2B vector. Secondly, the plasmids were sent for sequencing to confirm the sequence of the insert and restriction enzyme sites still available for recloning.

Maxiprep involves the extraction and purification of plasmid DNA in a large scale and the DNA yield is usually higher. For the amplification of plasmid DNA by maxiprep, transformation of competent *E. coli* DH5α was performed on kanamycin containing plates (described in materials and methods section). The DH5α cells that contain the recombinant plasmid were able to grow on kanamycin plates as the pCMV vector contained kanamycin resistance gene. Then transformation efficiency was calculated (Table 2A).

For good chemically competent cells, 10^5 to 10^6 cfu/ µg DNA are obtained. Both the plasmids were transformed efficiently. Higher transformation efficiency was observed in the pCMV-STX4$_{WT}$ plasmid. From the positive transformed colonies, plasmid DNA was isolated and its concentration and the purity were determined by the Nanodrop (ND 2000) reading (Table 2B). The extracted plasmids from the maxiprep need to have high concentration and high quality (purity). A total of 500ml volume of culture was used to isolate plasmid DNA.

The plasmid size was confirmed by single digestion with *Eco*RI and agarose gel electrophoresis (Figure 4.1). The pCMV-Tag-2B vector (vector map shown in materials section) consists of a number of restriction enzyme (RE) sites in the MCS region. So, the plasmid of interest is inserted in the MCS of the vector. The restriction enzymes E*co*RI, *Bam*HI, *Xho*I, *Hind*III and *Apa*I were used in the experiments either alone or in pairs- *Eco*RI/*Bam*HI, *Eco*RI/*Xho*I, *Bam*HI/*Xho*I and *Eco*RI/*Apa*I in order to determine the available restriction sites for recloning into other vectors.

The amplified plasmids after maxiprep were then single and double digested by restriction enzymes in order to confirm the constructs. The double digested plasmid is expected give a band of insert corresponding to around 897 bp of STX4$_{WT}$ and 370 bp of STX4$_{mut}$ along with vector backbone pCMV of 4.3 kb (Figure 4.2 and 4.3). Among the various restriction endonuclease used, *Hind*III showed some sort of star activity in both pCMV-STX4$_{WT}$ and pCMV-STX4$_{mut}$ while other restriction enzymes used E*co*RI, *Bam*HI, *Xho*I and *Apa*I cut the vector precisely.

In case of double digestion of vectors, the desired fall outs were obtained which corresponded to band size of 897 bp of STX4 $_{WT}$ and 370 bp of STX4$_{mut}$ in all the pairs of restriction enzymes used for digestion except in case of double digested pCMV-STX4 $_{WT}$ vector, where the restriction enzymes *Eco*RI/ *Bam*HI showed an unusual band. It might be due to star activity or contamination and was not further used for restriction digestion. Only the restriction enzymes that were able to cut the vector, giving band of insert were taken into consideration for further studies.

Table 2 (A): Determination of transformation efficiency

S. No.	Plasmid used	Amount of plasmid DNA(µg)	No. of colonies observed	Transformation efficiency
1	pCMV-STX4$_{WT}$	100x10^{-3}	1200	12x10^{6}
2	pCMV-STX4 STX4$_{mut}$	100x10^{-3}	300	3x10^{6}

Table 2 (B): Quantification and determination of purity of DNA by Nanodrop ND 2000

S. No.	Plasmid DNA	Concentration (ng/µl)	Purity ratio (A260/A280 $\geq$ 1.8)
1	pCMV-STX4$_{WT}$	17372.4 (17.37mg/ml)	1.99
2	pCMV-STX4$_{mut}$	20286.5 (20.28 mg/ml)	1.81

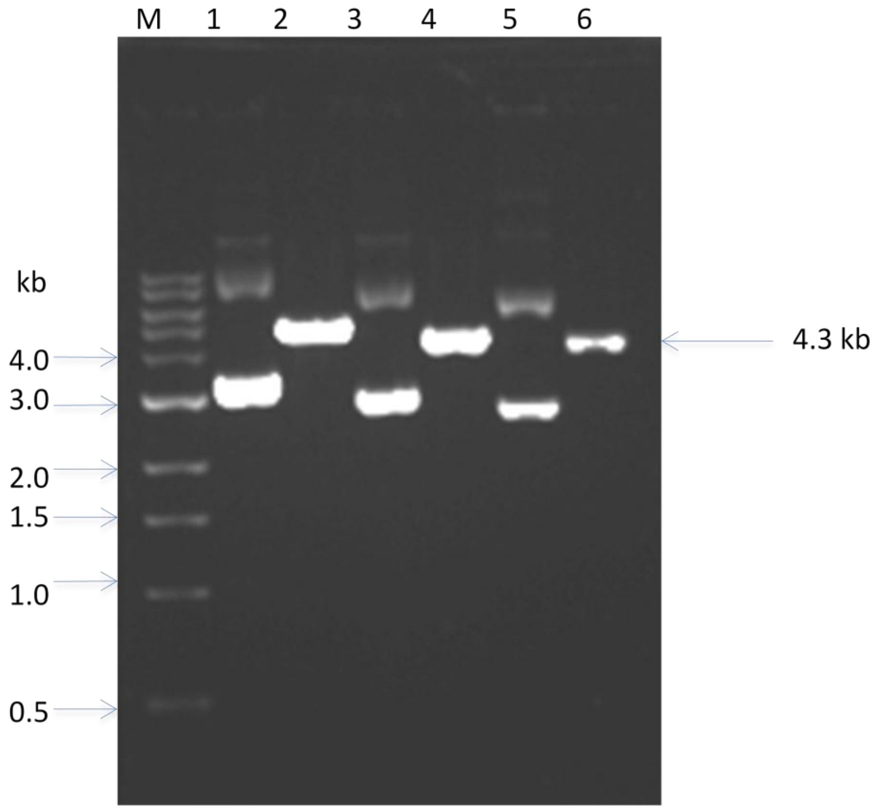

Figure 4.1: Agarose gel (0.6 %) electrophoresis of plasmid DNA digested with *Eco*RI restriction enzyme. The plasmids obtained from amplification by maxiprep, were digested with *Eco*RI and after performing 0.6% agarose gel electrophoresis, it was visualized under UV light in Gel Doc system (BIORAD). Lane 1, 3 and 5 undigested pCMV-STX4$_{wt}$, pCMV-STX4$_{mut}$ and pCMV-FLAG respectively. Lane 2 and 4 digested plasmids pCMV-STX4$_{WT}$ (5 kb) and pCMV-STX4$_{mut}$ (4.6 kb) respectively. The arrow (lane 6) indicates a linearized single digested vector fragment pCMV-FLAG without any insert (4.3 kb). M 1 kb NEB ladder.

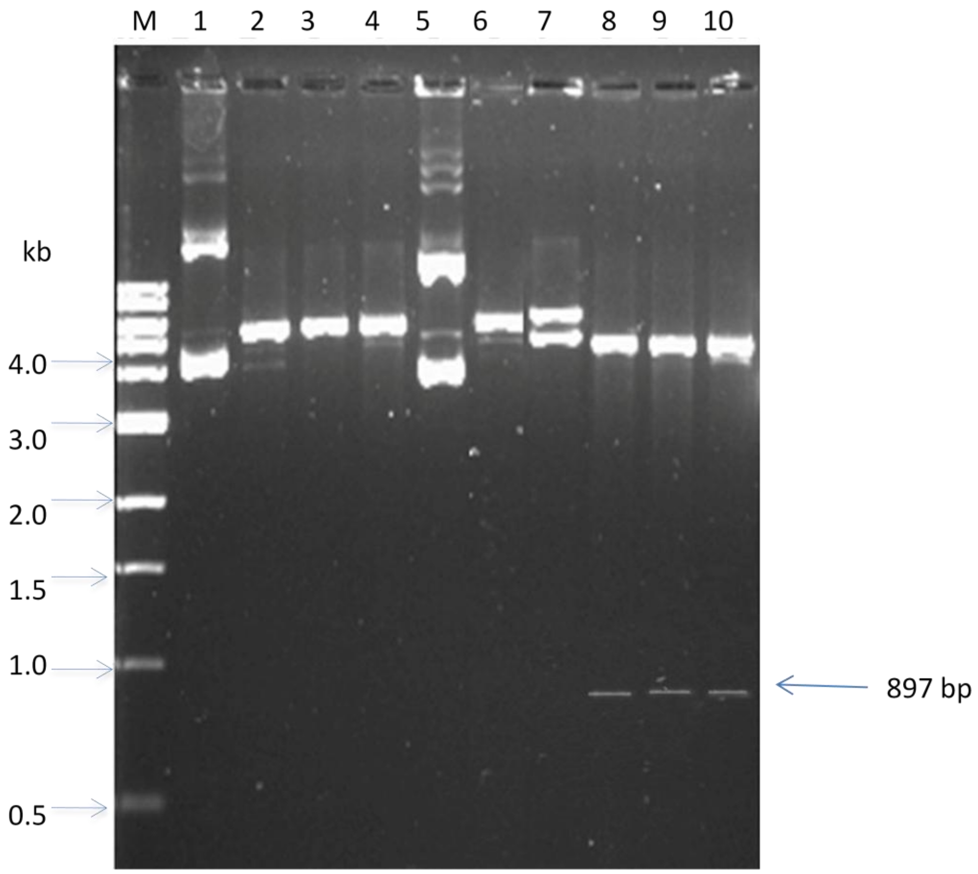

Figure 4.2: Agarose gel (0.8 %) electrophoresis of double digested plasmid pCMV-STX4$_{WT}$. Lane 1 undigested plasmid, Lane 2 to 6 plasmids digested with restriction enzymes *Eco*RI, *Bam*HI, *Xho*I, *Hind*III and *Apa*I respectively. Lane 7 to 10 double digested plasmid with pair of restriction enzymes *Eco*RI/*Bam*HI, *Eco*RI/*Xho*I, *Bam*HI/*Xho*I and *Eco*RI/*Apa*I respectively. The arrow indicates the band of insert corresponding to size of 897 bp on comparing with 1 kb NEB ladder M.

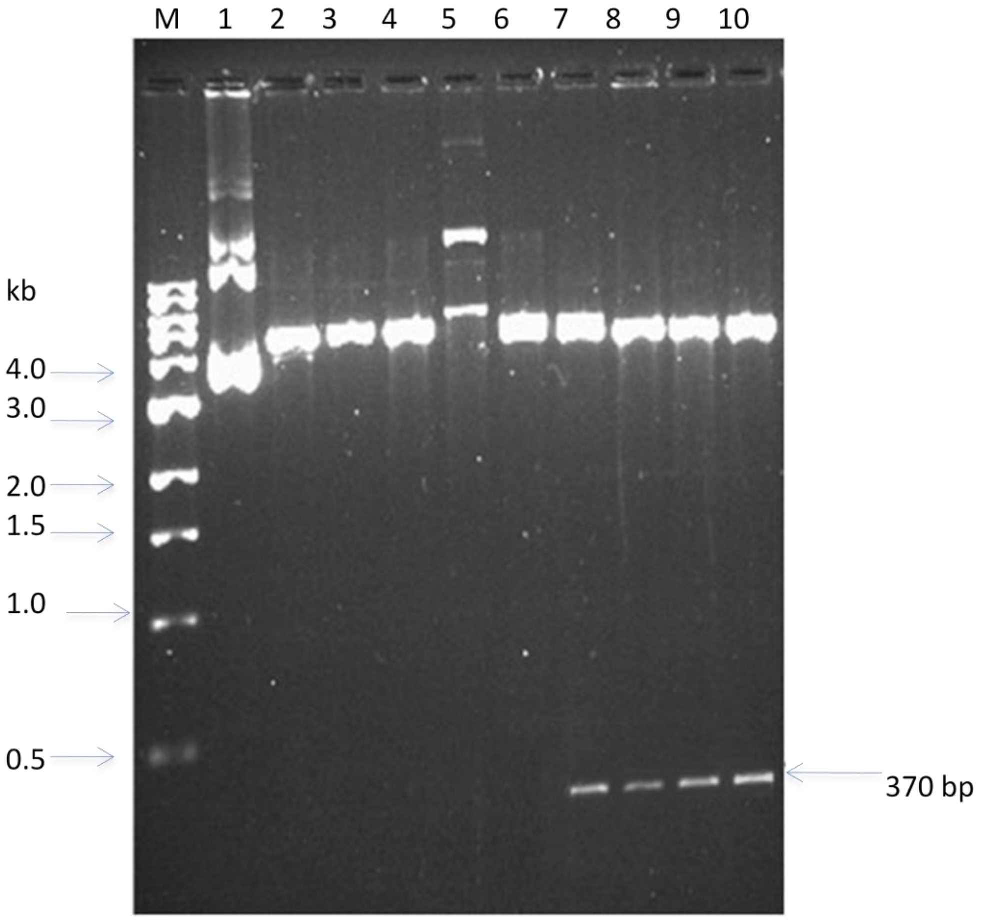

Figure 4.3: Agarose gel (0.8 %) electrophoresis of double digested plasmid pCMV-STX4$_{mut}$. Lane 1 undigested plasmid, Lane 2 to 6 plasmids digested with restriction enzymes *Eco*RI, *Bam*HI, *Xho*I, *Hind*III and *Apa*I respectively. Lane 7 to 10 double digested plasmid with pair of restriction enzymes *Eco*RI/*Bam*HI, *Eco*RI/*Xho*I, *Bam*HI/*Xho*I and *Eco*RI/*Apa*I respectively. The arrow indicates the band of insert corresponding to size of 370 bp on comparing with 1 kb NEB ladder M.

4.1.2 Sequence Analysis

After confirmation of the available RE sites on MCS of the pCMV-FLAG vector by double digestion, the plasmid DNA of cloned construct was sent for sequencing for the confirmation. For the analysis of sequences, the Rat STX4 coding sequence and amino acid sequence was retrieved from NCBI database.

The sequence obtained in each case- pCMV-STX4$_{WT}$ and pCMV-STX4$_{mut}$ using CMV Forward primer and pFastBac Reverse primer was analyzed and then aligned by MultAlin software for the sequence homology.

From the sequence alignment results of pCMV-STX4$_{WT}$, the obtained sequences were found to be highly consensus with the database sequence. The STX4$_{WT}$ was found to be inserted in between *Eco*RI and *Apa*I sites of the pCMV-Tag-2B vector, and this cloned construct contained an intact rat STX4 cDNA without any terminal deletion (Figure 4.4).

The obtained sequence for pCMV-STX4$_{mut}$ was found consensus at only specific regions, and totally non-homologous around the N-terminal regions of the insert. Hence the STX4$_{mut}$ was found to be truncated at the N-terminal region where the amino acids from 1-176 position were missing. So the truncated STX4 was containing only C-domain and referred to as N-terminal deleted STX4$_{mut}$ (Figure 4.5).

Rattus norvegicus syntaxin 4 (Stx4), mRNA-cds

NCBI Reference Sequence: NM_031125.1

>gi|13592098:32-928 Rattus norvegicus syntaxin 4 (Stx4), mRNA **897bp**

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CAGAAGGTGCAGACAATTCGGCAGACTATGGCCAAACTGGAGAGTAAAGTCCGGGAGTTGGA
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GAACCTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAAAGCCATAG
AGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCC
AGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAGTCCGA
GTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGT
GTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATAT
ACTGAAGGACACACAGGTGACCCGGCAGGCCCTGAATGAGATCTCTGCGCGACACAGTGAGAT
CCAGCAGTTGGAGCGCACGATCCGTGAACTCCATGAGATCTTCACTTTTCTAGCTACCGAGGTG
GAGATGCAGGGAGAGATGATCAATCGTATCGAAAAGAACATTCTGAGCTCAGCAGACTATGTG

GAACGTGGGCAAGAACATGTCAAGATAGCGCTAGAGAATCAGAAGAAGGCGAGGAAGAAAAA GGTCATGATTGCCATCTGTGTTTCTGTCACTGTTCTCATCTTGGCTGTCATCATTGGCATCACCAT AACCGTTGGATAA

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                    781    790       800       810       820       830       840       850       860       870       880       890       900       910
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 GACGATGAAGATGAGGTTCGAGTCGCGTTGGTGGTGCACTCAGGTGCCGCCCGGCTGAGCAGCCCGGACGACGAGTTCTTCCAGAAGGTGCAGACAATTCGGCAGACTATGGCCAAACTGGAGAGTAAAG
   Frsequencing(wild) GACGATGAAGATGAGGTTCGAGTCGCGTTGGTGGTGCACTCAGGTGCCGCCCGGCTGAGCAGCCCGGACGACGAGTTCTTCCAGAAGGTGCAGACAATTCGGCAGACTATGGCCAAACTGGAGAGTAAAG
  Revsequencing(wild)
           Consensus gacgatgaagatgaggttcgagtcgcgttggtggtgcactcaggtgccgcccggctgagcagcccggacgacgagttcttccagaaggtgcagacaattcggcagactatggccaaactggagagtaaag

                    911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 TCCGGGAGTTGGAGAAACAGCAGGTCACCATTCTGGCCACGCCTCTTCCCGAGGAGAGCATGAAGCAGGGCCTGCAGAACCTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAA
   Frsequencing(wild) TCCGGGAGTTGGAGAAACAGCAGGTCACCATTCTGGCCACGCCTCTTCCCGAGGAGAGCATGAAGCAGGGCCTGCAGAACCTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAA
  Revsequencing(wild)                                                                                 CTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAA
           Consensus tccgggagttggagaaacagcaggtcaccattctggccacgcctcttcccgaggagagcatgaagcagggcctgcagaacCTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAA

                    1041  1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 AGCCATAGAGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCCAGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAG
   Frsequencing(wild) AGCCATAGAGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCCAGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAG
  Revsequencing(wild) AGCCATAGAGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCCAGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAG
           Consensus AGCCATAGAGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCCAGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAG

                    1171  1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                    |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 TCCGAGTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGTGTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATA
   Frsequencing(wild) TCCGAGTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGTGTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATA
  Revsequencing(wild) TCCGAGTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGTGTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATA
           Consensus TCCGAGTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGTGTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATA

                    1301  1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
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      pCMVTag2B-Stx4 GATGCAGGGAGAGATGATCAATCGTATCGAAAAGAACATTCTGAGCTCAGCAGACTATGTGGAACGTGGGCAAGAACATGTCAAGATAGCGCTAGAGAATCAGAAGAAGGCGAGGAAGAAAAAGGTCATG
   Frsequencing(wild) GATGCAGGGAGAGATGATCAATCGTATCGAAAAGAACATTCTGAGCTCAGCAGACTATGTGGAACGTGGGCAAGAAC
  Revsequencing(wild) GATGCAGGGAGAGATGATCAATCGTATCGAAAAGAACATTCTGAGCTCAGCAGACTATGTGGAACGTGGGCAAGAACATGTCAAGATAGCGCTAGAGAATCAGAAGAAGGCGAGGAAGAAAAAGGTCATG
           Consensus GATGCAGGGAGAGATGATCAATCGTATCGAAAAGAACATTCTGAGCTCAGCAGACTATGTGGAACGTGGGCAAGAACatgtcaagatagcgctagagaatcagaagaaggcgaggaagaaaaaggtcatg

                    1561  1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680      1690
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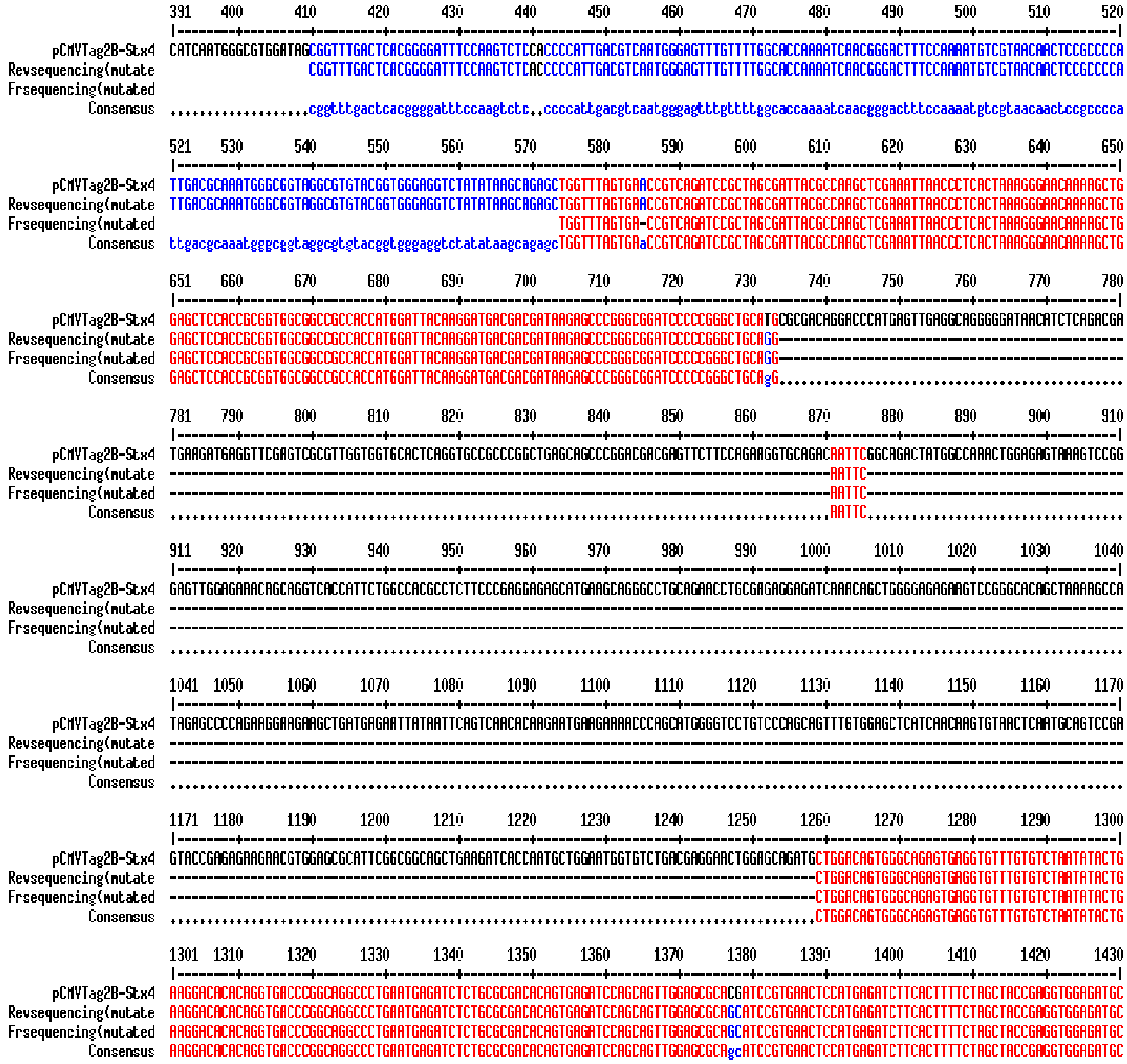
   Frsequencing(wild)
  Revsequencing(wild) ATTGCCATCTGTGTTTCTGTCACTGTTCTCATCTTGGCTGTCATCATTGGCATCACCATAACCGTTGGATAACTCGAGGGGGGGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTC
           Consensus attgccatctgtgtttctgtcactgttctcatcttggctgtcatcattggcatcaccataaccgttggataac..............ggtaccttaattaattaaggtaccaggtaagtgtacccaattc
```

Figure 4.4: Results of sequence alignment in pCMV-STX4$_{WT}$ by MultAlin



```
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Revsequencing(mutate                    CGGTTTGACTCACGGGGATTTCCAAGTCTCACCCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCA
Frsequencing(mutated
           Consensus ...................cggtttgactcacggggatttccaagtctc..ccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgcccca

                     521    530       540       550       560       570       580       590       600       610       620       630       640       650
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
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Revsequencing(mutate TTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTG
Frsequencing(mutated                                                      TGGTTTAGTGA-CCGTCAGATCCGCTAGCGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTG
           Consensus ttgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcagagcTGGTTTAGTGAaCCGTCAGATCCGCTAGCGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTG

                     651    660       670       680       690       700       710       720       730       740       750       760       770       780
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 GAGCTCCACCGCGGTGGCGGCCGCCACCATGGATTACAAGGATGACGACGATAAGAGCCCGGGCGGATCCCCCGGGCTGCATGCGCGACAGGACCCATGAGTTGAGGCAGGGGGATAACATCTCAGACGA
Revsequencing(mutate GAGCTCCACCGCGGTGGCGGCCGCCACCATGGATTACAAGGATGACGACGATAAGAGCCCGGGCGGATCCCCCGGGCTGCAGG-----------------------------------------------
Frsequencing(mutated GAGCTCCACCGCGGTGGCGGCCGCCACCATGGATTACAAGGATGACGACGATAAGAGCCCGGGCGGATCCCCCGGGCTGCAGG-----------------------------------------------
           Consensus GAGCTCCACCGCGGTGGCGGCCGCCACCATGGATTACAAGGATGACGACGATAAGAGCCCGGGCGGATCCCCCGGGCTGCAgG...............................................

                     781    790       800       810       820       830       840       850       860       870       880       890       900       910
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 TGAAGATGAGGTTCGAGTCGCGTTGGTGGTGCACTCAGGTGCCGCCCGGCTGAGCAGCCCGGACGACGAGTTCTTCCAGAAGGTGCAGACAATTCGGCAGACTATGGCCAAACTGGAGAGTAAAGTCCGG
Revsequencing(mutate ------------------------------------------------------------------------------------------AATTC-----------------------------------
Frsequencing(mutated ------------------------------------------------------------------------------------------AATTC-----------------------------------
           Consensus ..........................................................................................AATTC...................................

                     911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 GAGTTGGAGAAACAGCAGGTCACCATTCTGGCCACGCCTCTTCCCGAGGAGAGCATGAAGCAGGGCCTGCAGAACCTGCGAGAGGAGATCAAACAGCTGGGGAGAGAAGTCCGGGCACAGCTAAAAGCCA
Revsequencing(mutate ----------------------------------------------------------------------------------------------------------------------------------
Frsequencing(mutated ----------------------------------------------------------------------------------------------------------------------------------
           Consensus ..................................................................................................................................

                     1041  1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 TAGAGCCCCAGAAGGAAGAAGCTGATGAGAATTATAATTCAGTCAACACAAGAATGAAGAAAACCCAGCATGGGGTCCTGTCCCAGCAGTTTGTGGAGCTCATCAACAAGTGTAACTCAATGCAGTCCGA
Revsequencing(mutate ----------------------------------------------------------------------------------------------------------------------------------
Frsequencing(mutated ----------------------------------------------------------------------------------------------------------------------------------
           Consensus ..................................................................................................................................

                     1171  1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 GTACCGAGAGAAGAACGTGGAGCGCATTCGGCGGCAGCTGAAGATCACCAATGCTGGAATGGTGTCTGACGAGGAACTGGAGCAGATGCTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATATACTG
Revsequencing(mutate ----------------------------------------------------------------------------------------CTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATATACTG
Frsequencing(mutated ----------------------------------------------------------------------------------------CTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATATACTG
           Consensus ........................................................................................CTGGACAGTGGGCAGAGTGAGGTGTTTGTGTCTAATATACTG

                     1301  1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      pCMVTag2B-Stx4 AAGGACACACAGGTGACCCGGCAGGCCCTGAATGAGATCTCTGCGCGACACAGTGAGATCCAGCAGTTGGAGCGCACGATCCGTGAACTCCATGAGATCTTCACTTTTCTAGCTACCGAGGTGGAGATGC
Revsequencing(mutate AAGGACACACAGGTGACCCGGCAGGCCCTGAATGAGATCTCTGCGCGACACAGTGAGATCCAGCAGTTGGAGCGCAGCATCCGTGAACTCCATGAGATCTTCACTTTTCTAGCTACCGAGGTGGAGATGC
Frsequencing(mutated AAGGACACACAGGTGACCCGGCAGGCCCTGAATGAGATCTCTGCGCGACACAGTGAGATCCAGCAGTTGGAGCGCAGCATCCGTGAACTCCATGAGATCTTCACTTTTCTAGCTACCGAGGTGGAGATGC
           Consensus AAGGACACACAGGTGACCCGGCAGGCCCTGAATGAGATCTCTGCGCGACACAGTGAGATCCAGCAGTTGGAGCGCAgcATCCGTGAACTCCATGAGATCTTCACTTTTCTAGCTACCGAGGTGGAGATGC
```

Figure 4.5: Results of sequence alignment in pCMV-STX4$_{mut}$ by MultAlin.

4.2 Recloning of $STX4_{WT}$ and $STX4_{mut}$ into pEGFP-C2 and pDsRed1-C1 vectors

The sequences of inserts from pCMV-$STX4_{WT}$ and $STX4_{mut}$ and restriction sites from MCS were confirmed by sequencing. The inserts were found to be inserted in between *Eco*RI and *Apa*I sites of pCMV-FLAG (confirmed by double digestion also) and for the functional study of these plasmids, they were recloned into pEGFP-C2 and pdsRed1-C1 vectors. Directional cloning is a highly efficient method of directing the orientation of clonal inserts into vectors. By digestion of DNA insert or vector molecule with two restriction endonuclease enzymes, either blunt or non-complementary sticky ends at both ends of restriction fragment are created using restriction digestion. The insert can then be ligated to the vector plasmid in a specific, fixed orientation, without the risk of self-ligation by the cohesive ends during ligation.

4.2.1 Recloning into pEGFP-C2 vector

The purpose of cloning into EGFP vector is to be able to easily monitor transfection efficiency and localization of STX4. The strategy of cloning in the experiment is shown in Figure 4.6. In all cloning experiment, the cloning vector pEGFP-C2 and the source vector pCMV-Tag-2B containing the inserts ($STX4_{WT}$/ $STX4_{mut}$) were cut by the cloning enzymes (*Eco*RI and *Apa*I) and run on the gel. The linearized vector and the DNA fragments to be cloned were then extracted and purified.

The DNA was separated on 0.6% agarose gel (Figure 4.7) where the upper band indicated the vector backbone (pCMV-FLAG) while the lower indicated the fragment containing band of interest ($STX4_{WT}$ /$STX4_{mut}$). Similarly, the cloning vectors- pEGFP-C2/ pDsRed1-C1 were double digested and the upper linear dephosphorylated bands of vector on gel were cut out and purified using QIAGEN (QIAquick) gel extraction kit (described in section 3.15).

The purified insert and vector were run on 0.6% gel to determine the concentration of eluted fragments and then accordingly were ligated together (Figure 4.8). Typically, 50 ng of vector DNA (pEGFP-C2) and a fivefold molar excess of insert (calculated accordingly using formula described in section 3.16) were used with 200 units of Quick DNA Ligase (NEB) in a total volume of 20 µl at room temperature for 5-30 minutes. Half of the ligation mixture was immediately used to transform *E. coli* DH5α competent cells. After the transformation of *E. coli* cells with EGFP-$STX4_{WT}$ and EGFP-$STX4_{mut}$, a number of colonies were obtained. The transformation efficiency was calculated (Table 4) and was found to be lying within the range of 10^5 to 10^6 cfu/ µg DNA for both wild type and mutant Syntaxin 4.

In all cloning experiments, the plasmids were amplified by mini preparation (Alkaline Lysis method) and positive clones were randomly selected and screened using restriction enzyme digestion. Plasmid DNA was isolated from the positive clones surviving in kanamycin plates. The pair of restriction enzymes used in cloning i.e. *Eco*RI and *Apa*I was chosen for the digestion of the plasmid DNA EGFP-STX4$_{\text{WT}}$ obtained (Figure 4.9).The clones on gel that produced two 4.7 kb and 897 bp (fall out) bands after digestion were selected as positive clones and further amplified by maxiprep and sent for sequencing for the confirmation.

Similarly, plasmid DNA isolated from EGFP-STX4$_{\text{mut}}$ positive clones growing on the kanamycin containing plates were then digested with cloning enzymes- *Eco*RI and *Apa*I and subsequently analyzed on the gel (Figure 4.10). The clones on gel that produced two 4.7 kb and 370 bp (fall out) bands after digestion suggested that STX4$_{\textbf{mut}}$ gene has been successfully recloned in pEGFP-C2 vector. The positive clones were further amplified by maxiprep and sent to sequencing for confirmation.

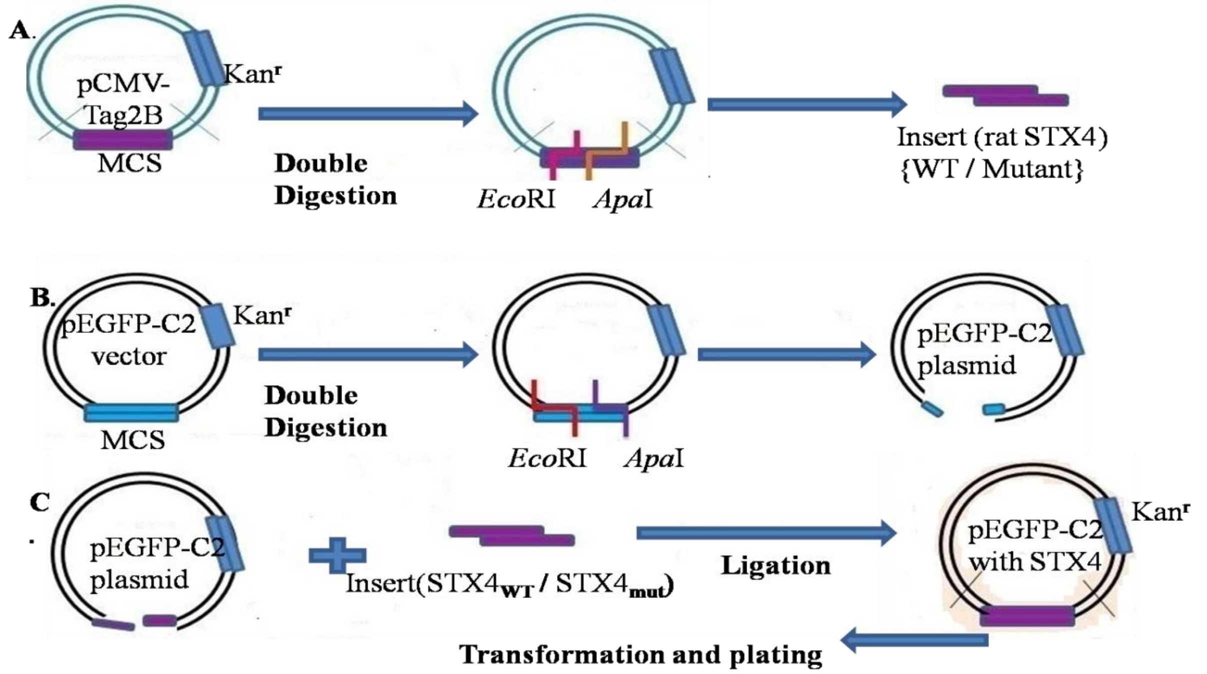


Figure 4.6: Schematic representation of cloning strategy of Rat STX4 $_{\text{WT}}$ and STX4$_{\text{mut}}$ in pEGFP-C2 expression vector.

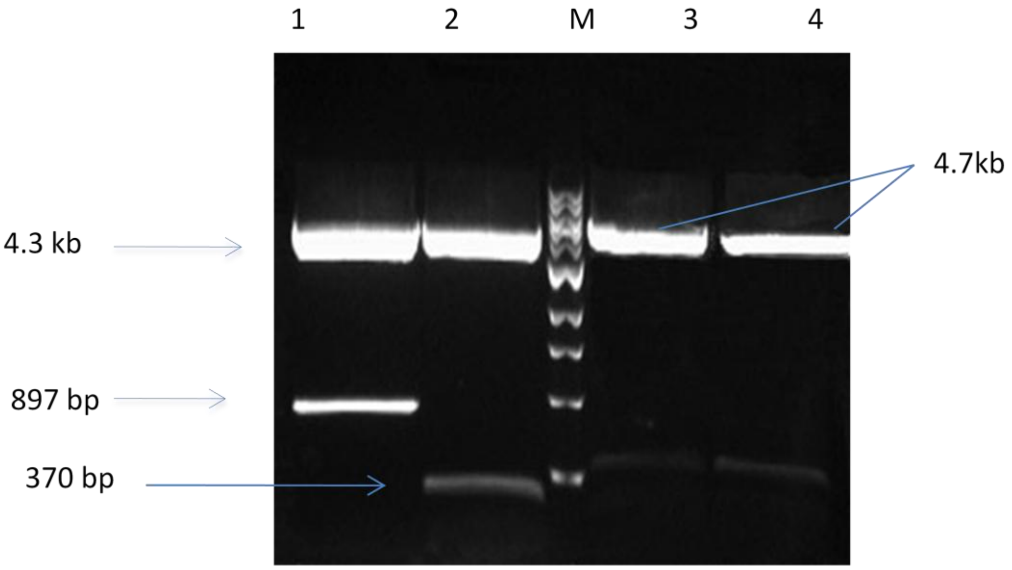


Figure 4.7: Extraction of STX4$_{WT}$, STX4$_{mut}$ insert and pEGFP-C2, pDsRed1-C1 vector fragments after *Eco*RI/*Apa*I double digestion. Taking 5 µg as template, source vector and cloning vectors were double digested with *Eco*RI and *Apa*I. Lane 1 and 2 double digested pCMV-STX4$_{WT}$ and pCMV-STX4$_{mut}$ showing 4.3 kb pCMV backbone and 897 bp and 370 bp insert band respectively. Lane 3 and 4 double digested vectors pEGFP-C2 and pDsRed1-C1 with vector fragment of 4.7 kb in each. M 1 kb NEB ladder.

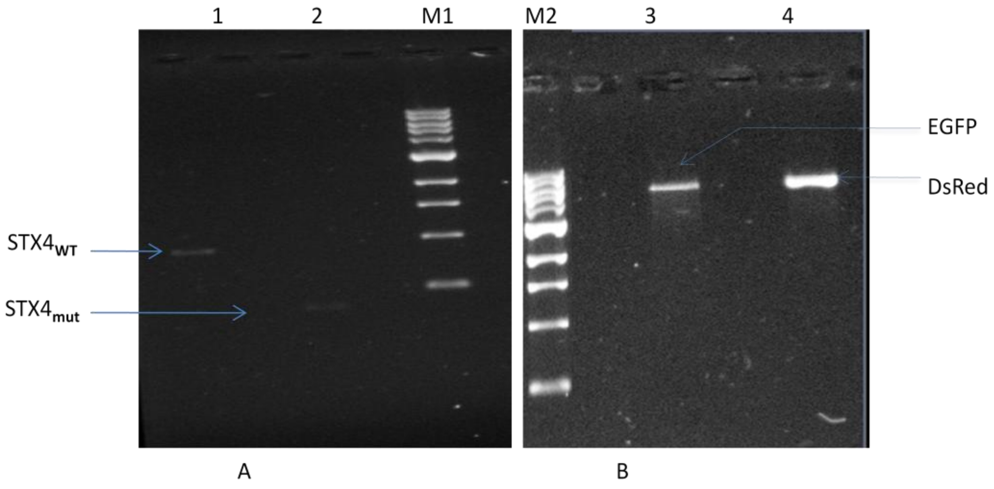


Figure 4.8: Agarose gel (0.6%) electrophoresis of eluted DNA fragments. Lane 1 and 2 (figure A) STX4$_{WT}$ and STX4$_{mut}$ eluted DNA fragment of 897 bp and 370 bp respectively. Lane 3 and 4(figure B) pEGFP-C2 and pDsRed1-C1 eluted DNA fragments of 4.7 kb band size each. M1 and M2 1 kb NEB DNA ladder.

Table 3: Quantification of eluted DNA fragments

S. No.	DNA fragment	Concentration (ng/µl)
1	$STX4_{WT}$	8 ng/µl
2	$STX4_{mut}$	5 ng/µl
3	pEGFP-C2	17 ng/µl
4	pDSRed1-C1	50 ng/µl

Table 4: Determination of transformation efficiency

S. No.	Plasmid used	Amount of plasmid DNA(µg)	No. of colonies observed	Transformation efficiency
1	EGFP-$STX4_{WT}$	$10x10^{-3}$	100	$10x10^{6}$
2	EGFP-$STX4_{mut}$	$10x10^{-3}$	150	$15x10^{6}$

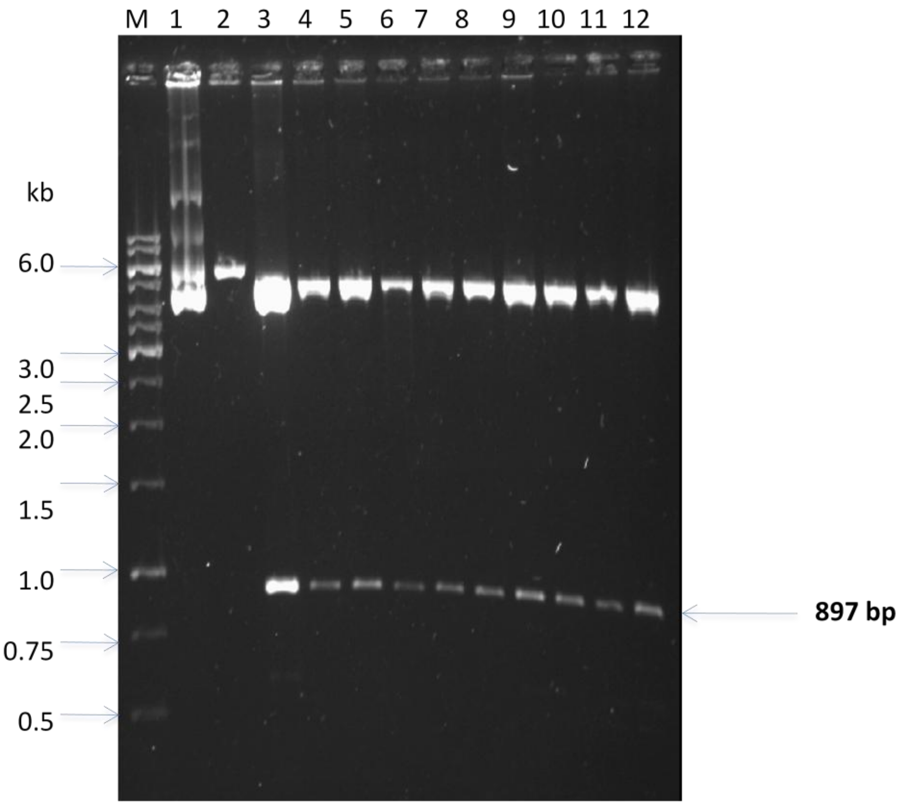


Figure 4.9: Restriction analysis of EGFP-STX$_{WT}$ clones by 0.6 % agarose gel electrophoresis. Lane 1 undigested plasmid, Lane 2 single digested plasmid with *Eco*RI. Lane 3 to 12 double digested plasmids with restriction enzymes *Eco*RI and *Apa*I. The arrow indicates the band of desired fallout corresponding to size of 897 bp in each clone. M 1 kb Gene Ruler DNA ladder.

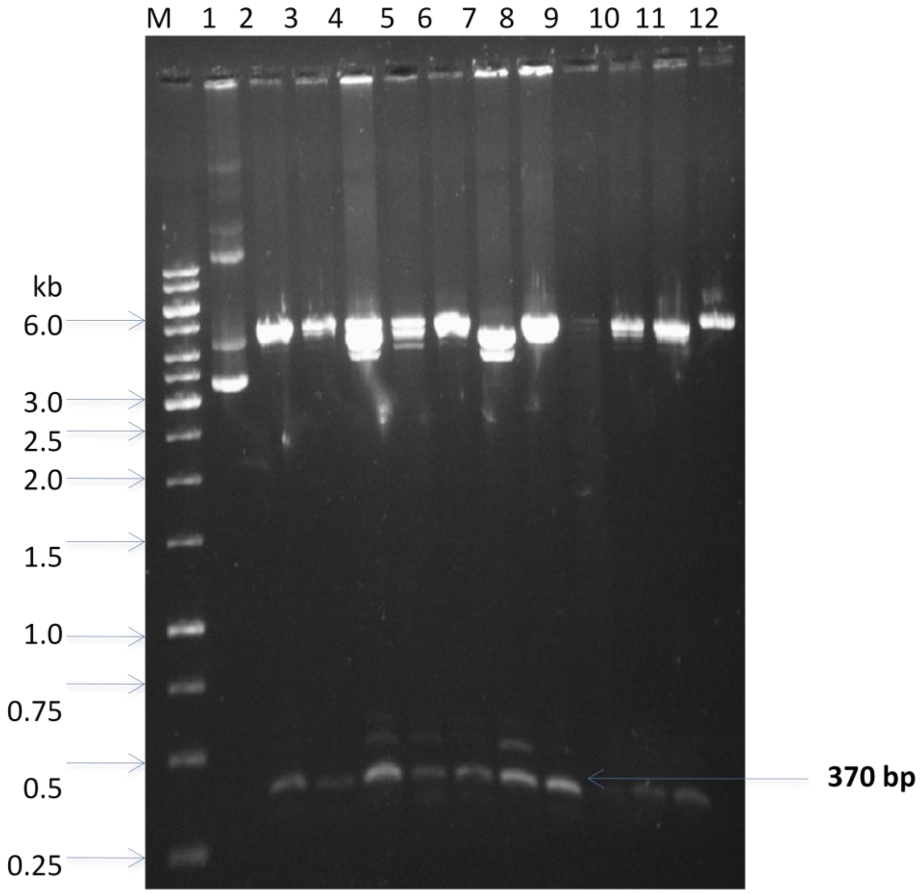


Figure 4.10: Restriction analysis of EGFP-STX$_{mut}$ clones by 0.6% agarose gel electrophoresis. Lane 1 undigested plasmid. Lane 2 to 11 double digested plasmids with restriction enzymes *Eco*RI and *Apa*I. Lane 12 single digested plasmid with *Eco*RI The arrow indicates the band of desired fallout of corresponding to size of 370bp. M 1kb Gene Ruler DNA ladder.

4.2.2 Recloning into pDsRed1-C1 vector

The $STX4_{WT}$ and $STX4_{mut}$ were also recloned into pDsRed1-C1 vector with the same strategy as in EGFP vector. The cloning vector pDsRed-C1 and the source vector pCMV-$STX4_{WT}$ and pCMV-$STX4_{mut}$ were cut by the appropriate restriction enzymes- *Eco*RI and *Apa*I and run on the 0.6% agarose gel (shown in Figure 4.7). The linear vector and the DNA fragments to be cloned were then extracted and purified as described previously.

The linearized vector and the insert fragments (Figure 4.9) were quantified (Table 3) and ligated accordingly with T4 DNA ligase. Half of the ligated mixture was used for transformation of *E. coli* DH5α competent cells and the colonies that were able to grow on kanamycin containing plates were counted.

Higher transformation efficiency was obtained in case of DsRed-$STX4_{mut}$ (Table 5). To identify the clones carrying the recombinant vector plasmid with the desired insert, colonies able to grow on kanamycin containing plate were picked, plasmid DNA extracted by standard mini preparation and analysed by restriction digestion.

The resulting double digested plasmids were then separated on 0.7% agarose gel to confirm whether the cloning of $STX4_{WT}$/ $STX4_{mut}$ into pDsRed1-C1 had been successful. In case of DsRed- $STX4_{WT}$, it was expected to see two bands, upper band of vector of 4.7 kb and lower band of $STX4_{WT}$ insert of 897 bp (Figure 4.11). And in DsRed- $STX4_{WT}$ clones, the double digested plasmids were expected to produce DNA fragments of 4.7 kb vector and 370 bp $STX4_{mut}$ (Figure 4.12).

Table 5: Determination of transformation efficiency

S. No.	Plasmid used	Amount of plasmid DNA(μg)	No. of colonies observed	Transformation efficiency
1	DsRed-$STX4_{WT}$	$10x10^{-3}$	5	$5x10^{5}$
2	DsRed-$STX4_{mut}$	$10x10^{-3}$	300	$30x10^{6}$

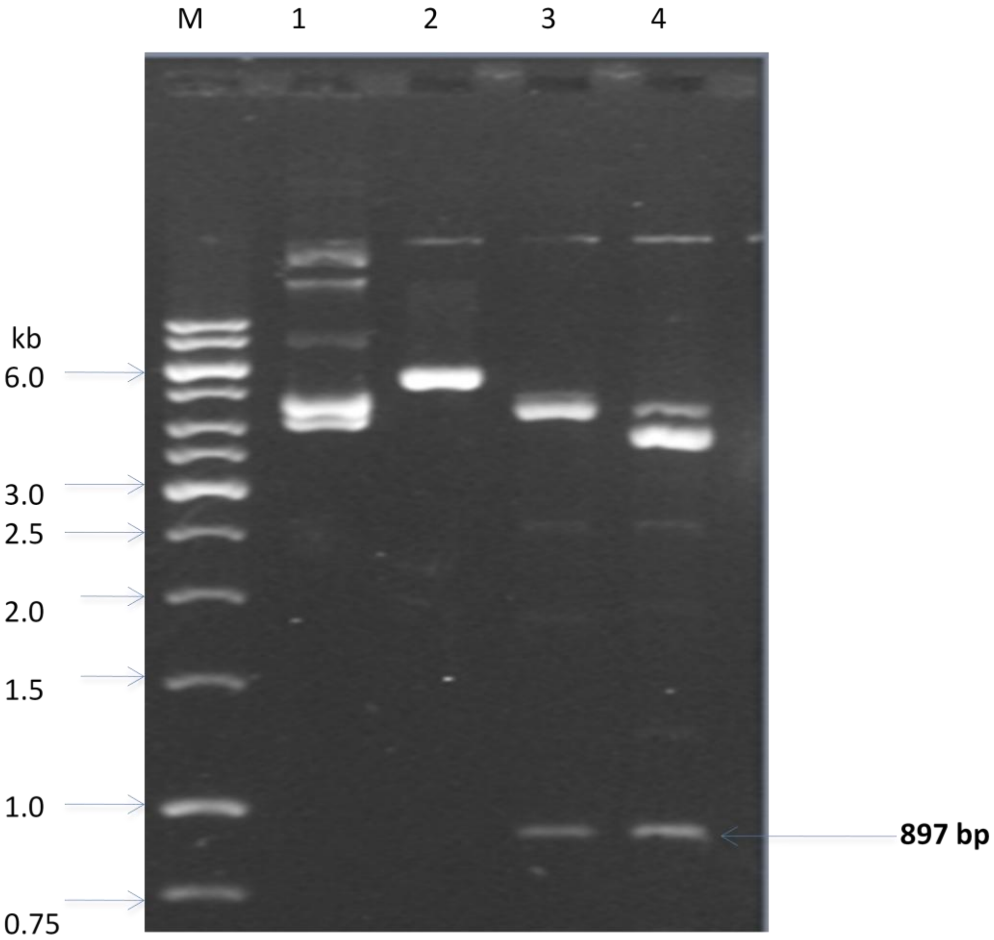


Figure 4.11: Agarose gel (0.6 %) electrophoresis of DsRed-STX$_{WT}$ clones digested with *Eco*RI and *Apa*I enzymes. Lane 1 undigested plasmid, lane 2 single digested plasmid with *Eco*RI. Lane 3 and 4 double digested plasmid with restriction enzymes *Eco*RI and *Apa*I. The arrow indicates the band of desired fallout corresponding to size of 897 bp. M 1 kb Gene Ruler DNA ladder.

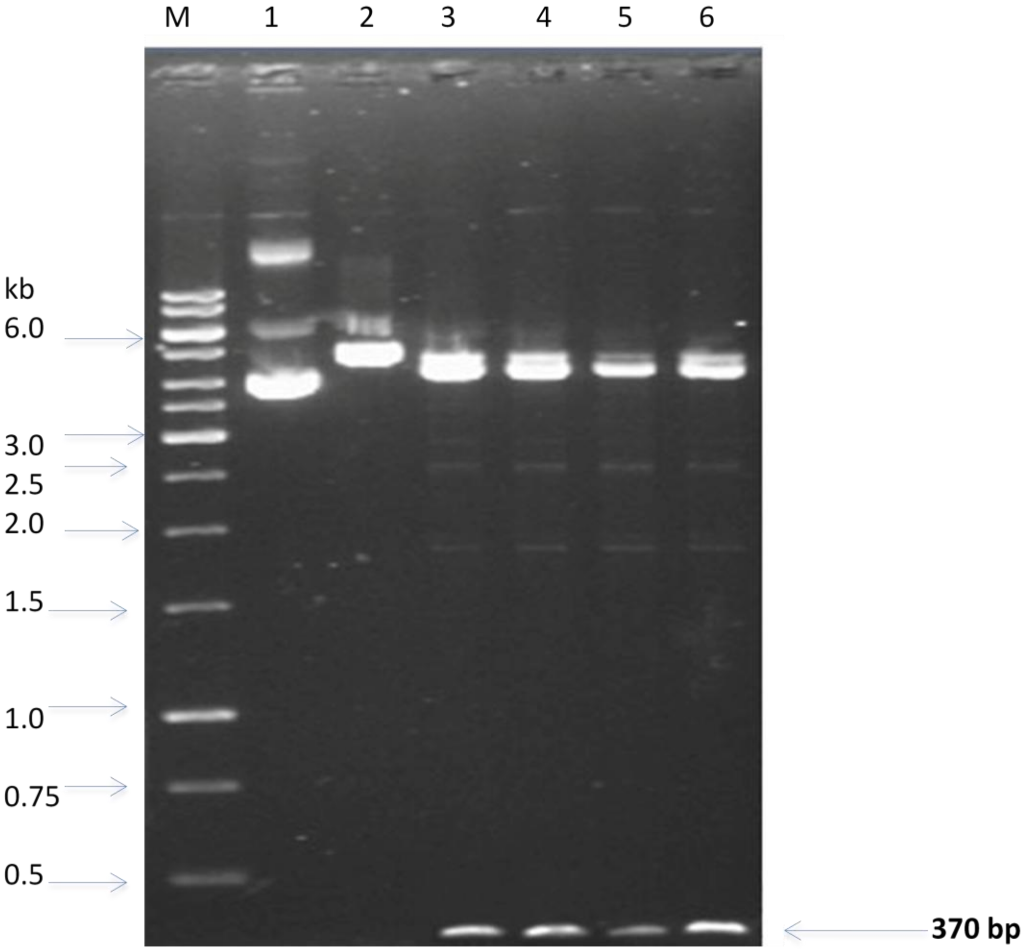


Figure 4.12: Agarose gel (0.6 %) electrophoresis of DsRed-STX$_{mut}$ clones digested with *Eco*RI and *Apa*I enzymes. Lane 1 undigested plasmid, lane 2 single digested plasmid with *Eco*RI. Lane 3 to 6 double digested plasmids with restriction enzymes *Eco*RI and *Apa*I. The arrow indicates the band of desired fallout of band size 370 bp. M 1 kb Gene Ruler DNA ladder.

4.3 Expression of FLAG/ EGFP-tagged $STX4_{WT}$ and $STX4_{mut}$ in RBL mast cells

4.3.1 Flowcytometric analysis to determine transfection efficiency

A high efficiency transient transfection protocol has been previously described (Puri *et al.,* 2003). Inorder to analyze transfection efficiency of RBL cells and maximize cell recovery, EGFP tagged plasmid (20 µg) were transfected into RBL cells. Cells were harvested 24 hours and percentage transfection efficiency was analyzed by flow cytometry for the expression of EGFP. The EGFP transfection efficiency was found to be 24.06% (Figure 4.13).

4.3.2 Effect of overexpression of $STX4_{WT}$ or $STX4_{mut}$ on mast cell exocytosis

To identify the regulatory role of N-terminal regulatory domain in the regulated exocytosis from the mast cells by overexpressing $STX4_{WT}$ or $STX4_{mut}$ on mast cells, in this study transient transfection approach was used in which the $STX4_{WT}$ and $STX4_{mut}$ were overexpressed along with an exocytosis reporter, human growth hormone(hGH). The mast cells do not express growth hormone, so the exocytosis due to transient transfection of the DNA of interest can be easily monitored by the hGH release (as described in Puri *et al.*, 2003). Transfected cells were stimulated by IgE cross-linking and the extent of degranulation was measured by analyzing the amount of hGH released from the cells. Release of about 40% of total hGH was observed from $STX4_{WT}$ transfected RBL mast cells. Expression of $STX4_{mut}$ slightly enhanced exocytosis from transiently transfected RBL mast cells (Figure 4.14).

4.3.3 Fluorescence microscopic analysis to determine the membrane localization of $STX4_{WT}$ and $STX4_{mut}$

The RBL cells cotransfected with EGFP tagged $STX4_{WT}$ or $STX4_{mut}$ were cultured on coverslips and observed under fluorescent microscope 24 hours after transfection . The EGFP tagged $STX4_{WT}$ localized primarily on the plasma membrane and RBL mast cells. EGFP tagged N-terminal deleted $STX4_{mut}$ did not show plasma membrane localization in RBL mast cells and was instead present internally inside the cells. The punctate pattern of GFP-tagged $STX4_{mut}$ is shown in Figure 4.15.

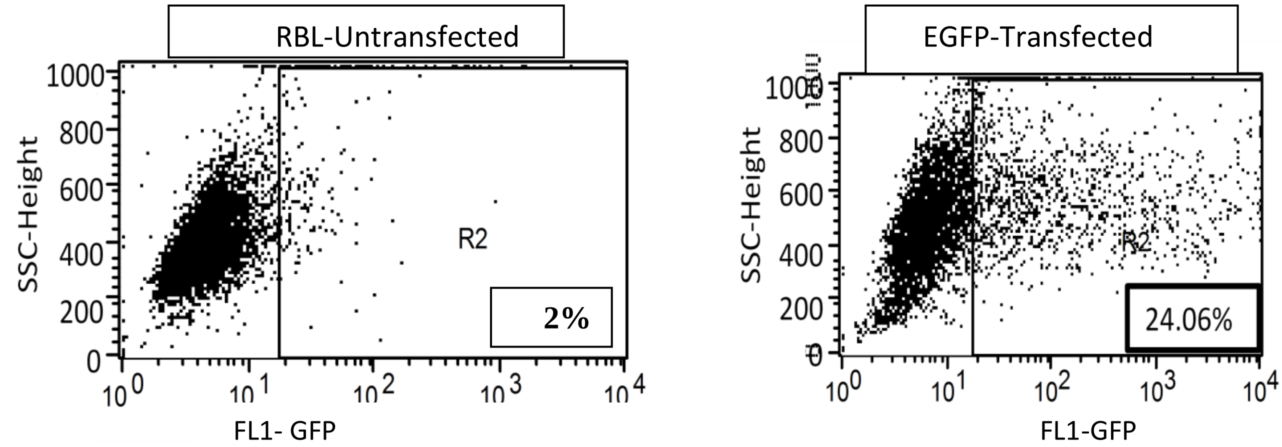
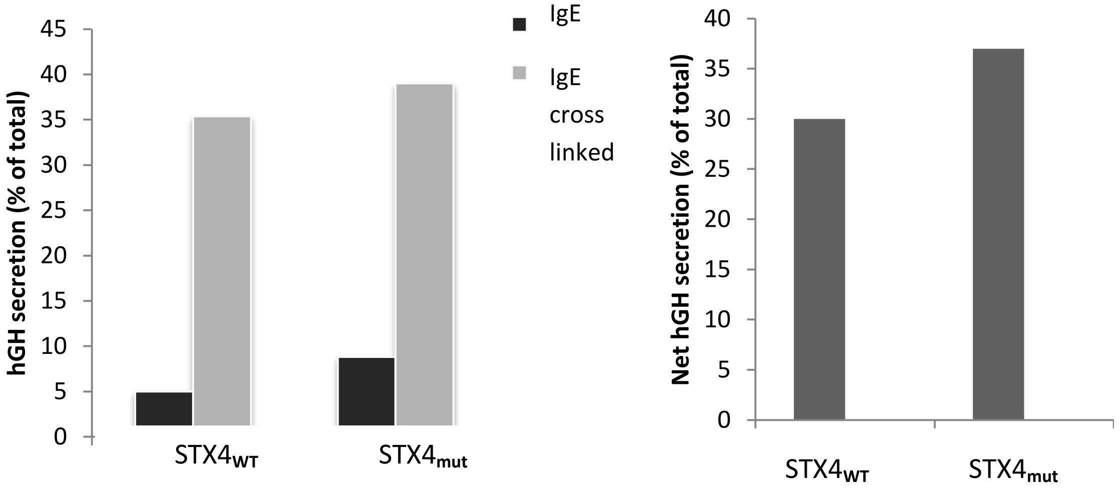


Figure 4.13: Analysis of transfections in RBL cells. 10 million RBL cells were transfected with 20 µg EGFP plasmid by electroporating at 320V and 950 µF. 24 hours post-transfection, cells were harvested and analyzed by flow cytometry.



A **B**

Figure 4.14: Release of hGH from transfected RBL mast cells. RBL cells co-transfected with 2µg hGH and 18µg of pCMV-STX4$_{WT}$ or STX4$_{mut}$ were plated in 6 well plate. Cells were stimulated for secretion by IgE crosslinking and after 16-18 hrs induced for secretion with anti IgE DNP-BSA for 45 min. Cell supernatant was collected and the cells were lysed in 0.2% Triton X-100 in RPMI PR$^-$ medium in shaking condition at 4^{o}C. Amount of hGH was measured in both supernatants and lysates by hGH ELISA. Figure A shows the amount of hGH released and calculated as a percentage of the total amount of hGH present in the cells. The net hGH secretion of the samples shown in A is shown in figure B.

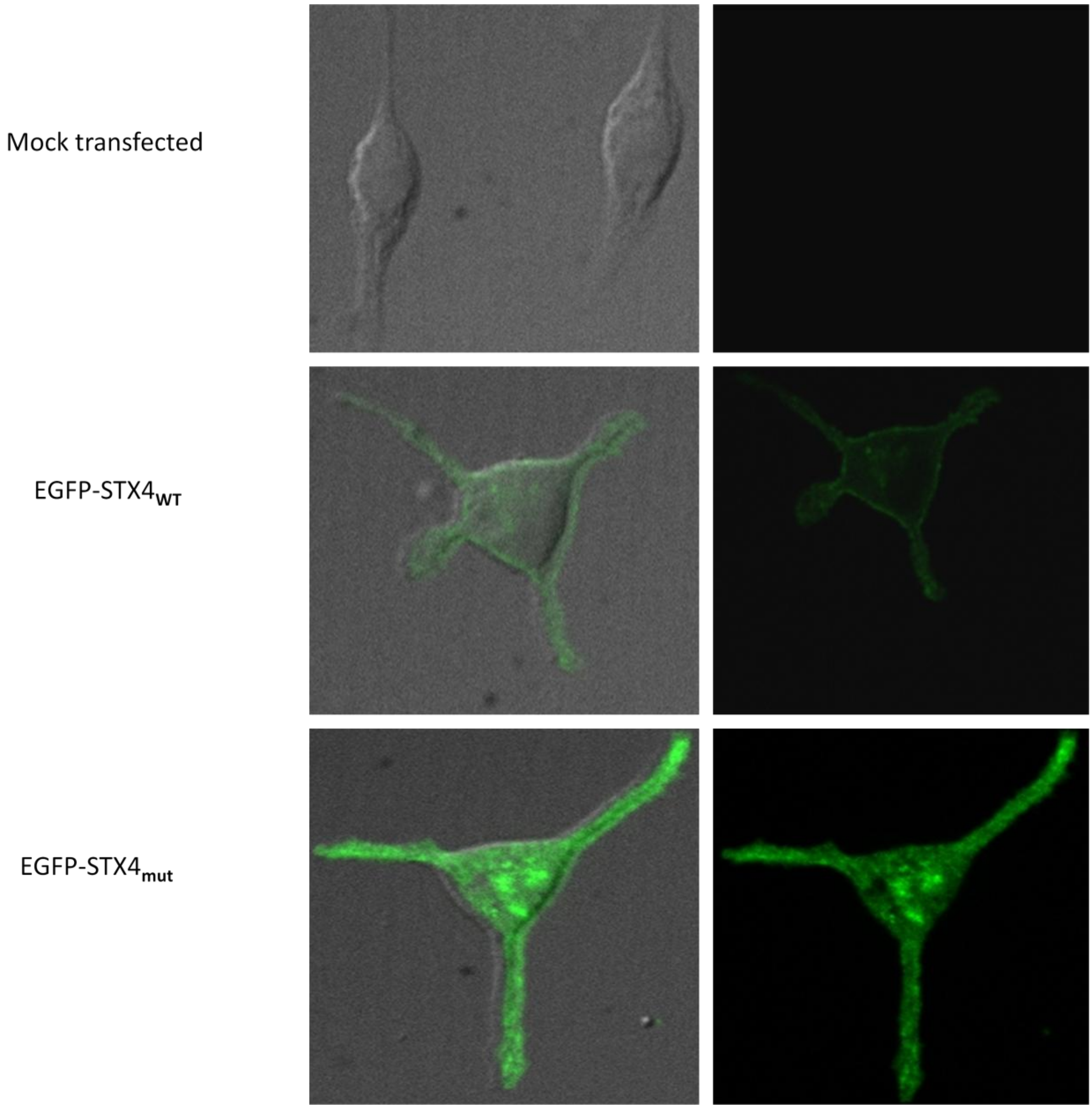


Figure 4.15: Expression of membrane targeting of EGFP tagged STX4$_{WT}$ and STX4$_{mut}$. RBL cells transfected with EGFP tagged STX4$_{WT}$ or STX4$_{mut}$ were cultured on coverslips. 24 hrs post-transfection, cells were fixed with 4% PFA. Coverslips were mounted in Fluoromount G and images were collected with Nikon Eclipse Ti microscope. All images were taken at 60X magnification. Shown here is one representative image in each case in different contrasts.

DISCUSSION

Regulated exocytosis is critical for the functioning of mast cells, which are major cell type mediating inflammatory and immune regulatory responses. SNARE mediated secretory granule/plasma membrane fusion is the essence of regulated exocytosis. The interacting SNARE partners (v-SNAREs on vesicle and t-SNAREs on target membrane) assemble through their coiled coil domains to form a trans-SNARE complex which is minimally required for membrane fusion. Several molecular techniques have been used for the study of SNARE machinery. Development of transient transfection method utilizing RNA interference technology to selectively target and down regulate SNARE protein, elucidates the importance of that protein. Knockout/ knockdown of a SNARE protein have been used to study its functional implications. Syntaxin 4 knockout has been used for the investigation of physiologic function of STX4 in the regulation of GLUT4 vesicle trafficking (Yang *et al.,* 2001). The knockout /knockdown may not be usable to the study of all SNARE proteins due to limited access of knockout mice and for this various cell lines have been used for the *in vitro* expression of SNARE proteins. Tagging of SNARE proteins with fluorescent tags like GFP and RFP helps in protein localization and visualization of cell responses. Also the tagging helps in studying of various interacting SNARE proteins in real time by live cell imaging.

It is well studied that N-terminal domain of Syntaxin plays an important role in exocytosis of synaptic vesicles in neurons, while there is little data regarding the importance of this domain in regulated exocytosis of nonneuronal cells such as mast cells. Previous studies have shown that Syntaxin 4 N-terminal is involved in complex formation with regulatory protein Munc-18c and it showed that N-terminal 29 amino acids of Syntaxin 4 is critical for this interaction (Latham *et al.,* 2006). For the localization and intercellular trafficking of interacting SNARE proteins, they can be expressed as GFP- and FLAG tagged fusion proteins in various cell lines. A study by Takuma *et al.,* 2002 showed that GFP-Syntaxin 4 was undetectable on the plasma membrane but was strongly expressed on unidentified unusually large vesicles. GFP-Syntaxin 4 without its transmembrane domain was still incompletely soluble and observed as aggregates (Takuma *et al.,* 2002). During this study, the main aim was to study the role of N-terminal regulatory domain of Syntaxin 4 in mast cell exocytosis. For this, the FLAG tagged Syntaxin 4 (wild type/ mutant) were cloned into EGFP and RFP vectors to provide them a fluorescent tag so that the proteins could be visualized. The EGFP tagged $STX4_{WT}$ was found to localize primarily on plasma membrane while the EGFP-tagged N-terminal deleted mutant, $STX4_{mut}$ did not show plasma membrane association in RBL mast cells and was instead localized within cytosol or may be associated in some organelles present internally inside the cells. Interestingly, the N-terminal regulatory domain has been found to have role in targeting also as the N-terminal deleted mutant was unable to target to the plasma membrane. This is a preliminary study and a lot more studies have to be done to confirm these results.

Recent studies have shown that binding of regulatory protein Munc-18c to Syntaxin 4 is required for the stabilization of Syntaxin 4 at the plasma membrane but not for its correct targeting. The N-terminus has been found to serve two functions in membrane targeting- first, it harbours the sorting motif which targets Synatxin 4 basolaterally to plasma membrane in Munc-18c- independent manner and second, it allows for Munc-18c dependent binding, which stabilizes the protein (Torres *et al.,* 2011).

The RBL mast cell line has been widely used as a model system for *in vitro* studies regarding mechanisms of mast cell degranulation. In this study, transient transfection approach was utilized in which full length $STX4_{WT}$ or N-terminal deleted $STX4_{mut}$ were transfected into RBL cells along with an exocytosis reporter i.e. hGH. hGH when introduced in rat neuroendocrine cells, it allowed easy identification of transfected cells and has elucidated the roles of various secretion mediators (Puri *et al.,* 2003). Likewise, co-transfected hGH into RBL cells, when stimulated to degranulate, gives secretion profile expressed as net and total hGH secretion in percentage. From the results, it was observed that overexpression of $STX4_{mut}$ caused higher secretion as compared to $STX4_{WT}$. The precise molecular mechanisms governing these factors remain to be known. The implication of Syntaxin 4 in degranulation has been confirmed by functional studies. A study by Paumet *et al.,* 2000 showed that overexpression of Syntaxin 4 by transient transfection significantly inhibited FcεRI mediated exocytosis in RBL cells. The mechanisms by which overexpression of wild type Syntaxin 4 inhibits regulated exocytosis is not completely known, yet it is postulated that overexpression of this Syntaxin may interfere the SNARE complex leading to aberrant interactions and blockade of recycling factors like NSF or SNAP proteins (Paumet *et al.,* 2000). Here in this study the Syntaxin 4 N-terminal deleted mutant displayed normal exocytosis inspite of their decreased membrane association as compared to wild type Syntaxin 4.

The discrepancy between membrane localization and biological activity of these Syntaxin 4 mutants may be in part or due to the presence of endogenous Syntaxin 4 or may suggest a complex, multi-component process for association of Syntaxin 4 to membrane and its recruitment to a biologically productive state. The cascade of signaling events that ultimately leads to mast cell degranulation is exceedingly complex, involving various proteins. Membrane microdomain (lipid rafts) association, protein-protein interactions and protein trafficking studies have to be further done with these mutants to account for their differential effects in RBL mast cells. Silencing or knocking down of endogenous Syntaxin 4 by siRNA or some such technique followed by overexpression of the wild type or mutant Syntaxin 4 and then observing the effect of expgenously transfected Syntaxin 4 mutants on RBL exocytosis may provide a clearer and unbiased results. A further study is required to determine the precise mechanistic details of the role of Syntaxin 4 and its N-terminal regulatory domain in mast cell exocytosis and also in the SNARE complex assembly.

CONCLUSION

Mast cells are key effectors in immunity and activation through IgE receptors (FcεRI) triggers degranulation with release of inflammatory mediators stored in abundant cytoplasmic granules. Mast cell degranulation requires SNARE proteins for membrane fusion. Although some of the central SNARE proteins involved in mast cell secretory events have been defined, gaps still persist in our understanding of the membrane fusion process. Mast cells express wide array of SNAREs and to date, described SNARE proteins in mast cells include the t-SNAREs SNAP-23 as well as Syntaxin 2, 3, 4 and 6 while VAMP include VAMP 2, 3, 4, 7 and 8. The preliminary data in this study shows that N-terminal regulatory domain is important for mast cell exocytosis regulation as well as localization of Syntaxin 4. Further studies are required to determine precise mechanistic details on the role of regulatory domain of Syntaxin 4 and its various mutants on mast cells exocytosis. Membrane-cytosol fractionation, immunobloting, co-immuoprecipitation with other interacting SNARE proteins, 2D electrophoresis are the possible studies that can be done for the elucidation of precise role of Syntaxin 4 in mast cell exocytosis and also of its various domains in Syntaxin 4 function in general.

Regulated exocytosis is thus a critical event in the activation of mediator-containing secretory immune and inflammatory cells. The understanding of molecular machinery of regulated exocytosis has important therapeutic potential as activation of these cell types is directly implicated in the exacerbation of allergic inflammation in diseases such as asthma. It is vital to investigate the mechanisms regulating mast cell exocytosis, with a goal being the identification of therapeutics and development of novel small molecules for the treatment of allergic and autoimmune diseases.

REFERENCES

Antonin W, Fasshauer D, Becker S, Jahn R and Schneider TR (2002) Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat Struct Biol*. **9**: 107-111

Benehamou M and Blank U (2010) Stimulus-secretion coupling by high-affinity IgE receptor: new developments. *FEBS Lett.* **584**: 4941-4948

Bennett MK, Calakos N and Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science*, **257**: 255-259

Blank U (2011) The mechanisms of exocytosis in mast cells. *Adv.Exp. Med.Biol,* **716**: 107–122

Blank U and Rivera J (2004) The ins and outs of IgE-dependent mast- cell exocytosis. *Trends Immunol,* **2**: 266–273

Bock JB, Mattern HT, Peden AA and Scheller RH (2001) A genomic perspective on membrane compartment organization. *Nature.* **409**: 839-841

Bonifacino JS and Glick BS (2004) The mechanisms of vesicle budding and fusion. *Cell*. **116**: 153-166

Brenner S (1974) The genetics of caenorhabditis elegans. *Genetics.* **77**: 71-94

Brunger AT and DelaBarre B (2003) NSF and p97/VCP: similar at first, different at last. *FEBS Lett.* **555** 126-133

Castle JD, Guo Z, Liu L (2002) Function of the t-SNARE SNAP-23 and secretory carrier membrane proteins (SCAMPs) in exocytosis in mast cells. *Mol Immunol.* **38** (16-18): 1337-13340

Chapman ER, Barton AS, Barton N and Jahn R (1994) *J Biol Chem.* 269: 27427-27432

Chen YA and Scheller RH (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*. **2**: 98-106

Chernomordik LV and Kozlov MM (2005) Membrane hemifusion: crossing a chasm in two leaps. *Cell*. **123**: 375-382

Cunningham RE (2010) Overview of flow cytometry and fluorescent probes for flow cytometry. *Methods in Molecular Biology*. **588**: 319-326

Dulubova I, Khvotchev M, Liu S, Huryeva I, Sudhof TC and Rizo J (2007) Munc18-1 binds directly to the neuronal SNARE complex. *Proc Natl Acad Sci U S A*. **104**: 2697-2702

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC and Rizo J (1999) A conformational switch in syntaxin during exocytosis: role of munc18. *Embo J*, **18**: 4372-4382

Dvorak AM and Morgan ES (1997) Diamine oxidase-gold enzyme affinity ultrastructural demonstration that human gut mucosal mast cells secrete histamine by piecemeal degranulation *in vivo. J Allergen Clin Immunol.* **99**: 812-820

Fasshauer D, Sutton RB, Brunger AT and Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci U S A*, **95**: 15781-15786

Fasshauer D, Grubmuller H, Jahn R and Seidel CAM (2003) Single-molecule fluorescence resonance energy transfer reveals a dynamic equilibrium between closed and open conformations of syntaxin 1. *Proc Natl Acad Sci USA*. **100**: 15516-15521

Fernandez I, Ubach J, Dulubova I, Zhang X, Sudhof TC and Rizo J (1998) Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell*. **94**: 841-849

Flaumenhaft R, Croce K, Chen E, Furie B and Furie BC (1999) Proteins of the exocytotic core complex mediate platelet α–granule secretion. *J Biol Chem*. **274**: 2492

Foster LJ, Yeung B, Mohtashami M, Ross K, Trimble WS and Klip A (1998) Binary interactions of the SNARE proteins Syntaxin-4, SNAP-23 and VAMP-2 and their regulation by phosphorylation. *Biochemistry.* **37**: 11089-11096

Freshney RI (2010) Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6th Edition, Wiley-Blackwell Publications

Fukuda M (2008) Membrane traffic in the secretory pathway: Regulation of secretory vesicle traffic by Rab small GTPases. *Cell Mol Life Sci.* **65**: 2801-2813

Gagescu R (2000) SNARE hypothesis 2000. *Nat Rev Mol Cell Biol*. **1**: 5

Guo Z, Liu L, Cafiso D and Castle D (2002) Perturbation of a very late step of regulated step of regulated exocytosis by a secretory carrier membrane protein (SCAM2)-derive peptide. *J Biol Chem.* **277** (38): 35357-35363

Guo Z, Turner C and Castle D (1998) Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell.* **94**: 537-548

Hanson PI, Heuser JE and Jahn R (1997) Neurotransmitter release-four years of SNARE complexes. *Curr Opin Neurobiol.* **7**(3): 310-315

Hata Y, Slaughter CA and Sudhof TC (1993) Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature*. **366**: 347-351

Hepp R, Puri N, Hohenstein AC, Crawford GL, Whiteheart SW and Roche PA (2005) Phosphorylation of SNAP-23 regulates exocytosis from mast cells. *J Biol Chem*. **280** (8): 6610-6620

Holt M, Varoqueaux F, Wiederhold K, Takamori S, Urlaub H, Fasshauer D and Jahn R (2006) Identification of SNAP-47, a novel Qbc-SNARE with ubiquitous expression. *J Biol Chem*, **281**: 17076-17083

Hong W (2005) SNAREs and traffic. *Biochim Biophys Acta*. **1744**: 120–144

Jahn R and Scheller RH (2006) SNAREs-engines for membrane fusion. *Nat Rev Mol Cell Biol*. **7**: 631-643

Jahn R (2004) Principles of exocytosis and membrane fusion. *Ann NY Acad Sci*. **1014**: 170–178

Jahn R, Lang T and Sudhof TC (2003) Membrane Fusion (Review). *Cell*. **112**: 519-533

Kozak M (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem*. **266** (30): 19867-19870

Krutzik PO, Irish JM, Nolan GP and Perez OD (2004) Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clinical Immunology*. **110** (3): 206–221

Lacy P, Logan MR, Bablitz B and Moqbel R (2001) Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-–induced piecemeal degranulation in human eosinophils from atopic individuals. *J Allergy Clin Immunol.* **107**:671-678

Lane SR and Liu Y (1997) Characterization of the palmitoylation domain of SNAP-25. *J Neurochem*, **69**: 1864-1869

Latham CF, Lopez JA, Hu SH, Lee CL, Westbury E, Blair DH, Armishaw CJ, Alewood NJB, James DE and Martin JL (2006) Molecular dissection of the Munc18c/ Syntaxin4 interaction: implications for regulation of membrane trafficking. *Traffic.***7**: 1408-1419

Lin RC and Scheller RH (2000) Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev Biol*. **16**: 19-49

Lippert U, Ferrari DM and Jahn R (2007) Endobrevin/VAMP-8 mediates exocytotic release of hexosaminidase from rat basophilic leukemia cells. *FEBS Lett*, **581**: 3479-3484

Littleton JT, Chapman ER, Kreber R, Garment MB, Carlson SD and Ganetzky B (1998) Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron.* **21***:* 401

Logan MR, Odemuyiwa SO, Moqbel R (2003) Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. *J Allergy Clin Immunol.* **111**: 923- 932

Lorentz A, Baumann A, Vitte J and Blank U (2012) The SNARE machinery in mast cell secretion. *Frontiers in Immunology*. **143** (3): 1-13

Malsam J, Kreye S and Sollner TH (2008) Membrane fusion: SNAREs and regulation. *Cell Mol Life Sci.* **65**: 2814-2832

McNew JA, Parlati F, Fukuda R, Johnston RJ, Paz K, Paumet F, Sollner TH and Rothman JE (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature*. **407**: 153-159

Metcalfe DD, Baram D and Mekori YA (1997) Mast cells. *Physiol Rev.* **77** (4): 1033-1079

Misura KM, Scheller RH and Weis WI (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature*. **404**: 355-362

Moqbel R and Lacy P (1999) Exocytotic events in eosinophils and mast cells. *Clin Exp Allergy*. **29**:1017-22

Novick P and Zerial M (1997) The diversity of Rab proteins in vesicle transport. *Curr Opin cell biol.* **9**: 496-504

Novick P and Schekman R (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. **76**: 1858-1862

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE and Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J Cell Biol*. **109**: 3039-3052

Pang J, Zeng X, Xiao R, Lakatta EG and Lin L (2009) Design, generation, and testing of mammalian expression modules that tag membrane proteins. *Proteinscience.org.* Blackwell.

Paumet F, Rahimian V and Rothman JE (2004) The specificity of SNARE-dependent fusion is encoded in the SNARE motif. *Proc Natl Acad Sci USA*. **101**: 3376-3380

Paumet F, Le Mao J, Martin S, Galli T, David B, Blank U and Rao M (2000) Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8 containing secretory compartment. *J Immunol*. **164**: 5850-5857

Pombo I, Rivera J and Blank U (2003). Munc18- 2/syntaxin3 complexes are spatially separated from syntaxin3-containing SNARE complexes. *FEBS Lett*. **550**: 144–148

Prasher DC (1995) Using GFP to see the light. *Trends Genet.* **11**: 320-323

Puri N and Roche PA (2008) Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *PNAS*. **105** (7): 2580-2585

Puri N and Roche PA (2006) Ternary SNARE Complexes Are Enriched in Lipid Rafts during Mast Cell Exocytosis. *Traffic.* **7**: 1482-1494

Puri N, Kruhlak MJ, Whiteheart SW and Roche PA (2003) Mast cell Degranulation requires N-Ethylmaleimide sensitive factor mediated SNARE disassembly. *J Immunol*. **171**: 5345-5352

Ravichandran V, Chawla A and Roche PA (1996) Identification of a novel syntaxin and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J Biol Chem*. **271**: 13300-13303

Rickman C, Medine CN, Bergmann A and Duncan RR (2007) Functionally and spatially distinct modes of munc18-syntaxin 1 interaction. *J Biol Chem*. **282**: 12097-12103

Rivera J and Gilfillan AM (2006) Molecular regulation of mast cell activation. *J. Allergy Clin. Immuno,* **117**: 1214–1225

Rizo J and Sudhof TC (2002) SNAREs and Munc18 in synaptic vesicle fusion. *Nat Rev neurosci.* **3** (8): 641-653

Roa M, Paumet F, Lemao J, David B and Blank U (1997) Involvement of the ras like GTPase rab3d in RBL-2H3 mast cell exocytosis following stimulation via high affinity IgE receptors (FcepsilonRI). *J. Immunol.* **159**:2815–2823

Rossi V (2004) Longins and their longin domains: regulated SNAREs and multifunctional SNARE regulators. *Trends Biochem Sci.* **29**: 682-688

Sagi-Einsberg R (2007) The mast cell: where endocytosis and regulated exocytosis meet. *Immunol Rev.* **217**: 292-303

Salaun C and Chamberlain LH (2006) Lipid rafts as regulators of SNARE activity and exocytosis. *Molecular Mechanisms of Exocytosis.* **7**: 85-95

Sander LE, Frank SP, Bolat S, Blank U, Galli T, Bigalke H, Bischoff SC and Lorentz A (2008) Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activationinduced degranulation of mature human mast cells. *Eur J Immunol*. **38**: 855–863

Sayed BA, Christy A, Quirion MR and Brown MA (2008) The master switch: the role of mast cells in autoimmunity and tolerance. *Annu Rev Immunol.* **26**: 705-739

Shaphiro H (2003) Practical Flow Cytometry, Wiley-Liss, NY, USA

Shorter J, Beard MB, Seemann J, Dirac- Svejstrup AB and Warren G (2002) Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle- tethering protein. *J Cell Biol.* **157**: 45-62

Shukla A, Berglund L, Nielsen LP (2001). Regulated exocytosis in immune function: are SNARE proteins involved? *Respir Med*. **95**: 773–80

Sinha R, Ahmed S, Jahn R, and Klingauf J (2011) Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses. *Proc. Natl. Acad. Sci. USA*. **108**: 14318–14323

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P and Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**: 318-324

Stow JL, Manderson AP, Murray RZ (2006) SNAREing immunity: the role of SNAREs in the immune system. *Nat Rev*. **6**: 919–929

Steegmaier M, Yang B, Yoo JS, Huang B, Shen M, Yu S, Luo Y and Scheller RH (1998) Three novel proteins of the syntaxin/SNAP-25 family. *J Biol Chem*. **273**: 34171-34179

Sudhof TC and Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science.* **323**: 474–477

Sudhof TC (2004) The synaptic vesicle cycle. *Annu Rev Neurosci.* **27**: 509-547

Sutton RB, Fasshauer D, Jahn R and Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. **39**: 347-353

Suzuki K and Verma IM (2008) Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation. *Cell.* **134**: 485-495

Tadokoro S, Nakanishi M and Hirashima N (2005) Complexin II facilitates exocytotic release in mast cells by enhancing Ca^{++} sensitivity of the fusion process. *J Cell Sci*. **118**: 2239–2246.

Takuma T, Arakawa T, Okayama M, Mizoguchi I, Tanimura A and Tajima Y (2002) Trafficking of Green Fluorescent Protein-tagged SNARE proteins in HSY cells. *J Biochem.***132**: 729-735

Tellam, JT, McIntosh S and James DE (1995) Molecular identification of two novel Munc-18 isoforms expressed in non-neuronal tissues. *J Biol Chem*. **270**: 5857-5863

Teng FY, Wang Y and Tang BL (2001) The syntaxins. *Genome Biol*, **2**: REVIEWS3012

Tiwari N, Wang CC, Brochetta C, Scandiuzzi L, Hong W (2009) Increased formation of VAMP-3-containing SNARE complexes in mast cells from VAMP-8 deficient cells. *Inflamm Res*. **58**: 513–514

Trimble WS, Cowan DM and Scheller RH (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci U S A*. **85**: 4538-4542

Trun N and Trempy J (2003) Fundamental bacterial genetics. USA, Blackwell

Torres J, Funk HM, Zegers MMP and Beest MBA (2011) The syntaxin 4 N terminus regulates its basolateral targeting by Munc18c-dependent and independent mechanisms. *J.Biol.Chem*. **286**: 10834-10846

Tuvim MJ, Mospan AR, Burns KA (2009) Synaptotagmin 2 couples mucin granule exocytosis to Ca^{++} signaling from endoplasmic reticulum. *J Biol Chem*. **284**: 9781-9787

Vaidyanathan VV, Puri N and Roche PA (2001) The last exon of SNAP-23 regulates granule exocytosis from mast cells. *J Biol Chem*. **276**: 25101-25106

Valdez-Taubas J and Pelham H (2005) Swf-1 dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation. *EMBO J.* **24** (14): 2524-2532

Vogel K and Roche PA (1999) SNAP-23 and SNAP-25 are palmitoylated in vivo. *Biochem Biophys Res Commun*. **258**: 407-410

Wasiuk A, Vries VC de, Hartman K, Roers A and Noelle RJ (2009) Mast cells as regulators of adaptive immunity to tumours. *Clin Exp Immunol.* **155** (2): 140-146

Whitaker M (2000) Fluorescent tags of protein function in living cells. *Bioassays*. **22**: 180-187

Woska JR Jr, Gillespie ME (2012) SNARE complex-mediated degranulation in mast cells. *J Cell Mol Med*. **16**: 649–656

Woska JR Jr, Gillespie ME (2011) Small interfering RNA-mediated identification and regulation of the ternary SNARE complex mediating RBL-2H3 mast cell degranulation. *Scand J Immunol*. **73**: 8–17

Xu Y, Zhang F, Su Z, McNew JA and Shin YK (2005) Hemifusion in SNARE-mediated membrane fusion. *Nat Struct Mol Biol*, **12**: 417-422

Yang C, Coker KJ, Kim JK, Mora S, Thurmond DC, Davis AC, Yang B, Williamson RA, Shulman GI and Pessin JE (2001) Syntaxin 4 heterozygous knockout mice develop muscle insulin resistance. *J Clin Invest.* **107** (10): 1311-1318

Yang B, Gonzalez L Jr, Prekeris R, Steegmaier M, Advani RJ and Scheller RH (1999) SNARE interactions are not selective: implications for membrane fusion specificity. *J. Biol. Chem.* **274***:*5649-5653

Zerial M and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol*. **2**: 107-117

Zhou P, Pang ZP, Yang X, Zhang Y, Rosenmund C, Bacaj T, and Sudhof TC (2013a) Syntaxin-1 N-peptide and Habc-domain perform distinct essential functions in synaptic vesicle fusion. *EMBO J*. **32**: 159–171

Zhou P, Bacaj T, Yang X, Pang ZP and Sudhof TC (2013b) Lipid-Anchored SNAREs lacking transmembrane regions fully support membrane fusion during neurotransmitter release. *Neuron*. **470**: 470-483

APPENDICES

- **PBS (Phosphate Buffer Saline): 1 Liter**

NaCl	8 gm
$Na_2HPO_4.2H_2O$	1.44 gm
KCl	0.2 gm
KH_2PO_4	0.2 gm

pH was maintained to 7.3 to 7.4 with HCl

- **Iscover Media (Iscoves modified Dulbeccos Medium-Gibco/ Invitrogen, Catalogue no.- 12200-036): 1 Liter**

Glutamine	3 gm
$NaHCO_3$	2.6 gm
Gentamycin	120 mg

pH maintained to 7.0

- **MEM media (Minimum Essential Medium Eagle- Sigma, Catalogue no.- M0643): 1 Liter**

Glutamine	0.3 gm
$NaHCO_3$	2.6 gm
HEPES	2.5 gm
Gentamycin	120 mg

(pH- 7.25)

RPMI (Roswell Park Memorial Institute) complete medium: 1Liter

Glucose	1.0 gm
HEPES	2.50 gm
$NaHCO_3$	2.60 gm
L-Glutamine	0.30 gm
RPMI (SIGMA ALDRICH, USA)	10.40 gm
Gentamycin	120 mg

(pH- 7.0)

Luria Bertaini (LB) broth: 100 ml

Casein enzymatic hydrolysate	1 gm
Yeast extract	0.5 gm
NaCl	1 gm

(pH- 7.5)

2.5 gm of LB powder dissolved in 100ml Mili Q, autoclaved at 15 lb/in^2. For solid media, 1.5 % agar was added

SOC medium (Super Optimal Broth with Catabolite Repression): 100ml

Tryptone	2 gm (2%)
Yeast extract	500 mg (0.5%)
NaCl	50 mg (10 mM)

KCl	18.6 mg (2.5 mM)
MgCl2	95.2 mg (10 mM)
Glucose	360 mg (20 mM)

The final volume was made up to 100 ml with MQ H_2O, filter sterilized and stored at 4°C.

- ## Antibiotic Stocks:

Kanamycin	50 mg/ml
Ampicillin	100 mg/ml

The solutions were filter sterilized and stored at -20^{o}C.

- ## STE Solution:

0.1M NaCl

10mM Tris-Cl (pH-8)

1mM EDTA (pH 8)

The solution autoclaved at 15 lb/in^2 for 20 min and stored at 4°C.

- ## Solution I for plasmid isolation:

50 mM Glucose

25 mM Tris Cl (pH-8)

10 mM EDTA(pH 8)

The solution autoclaved at 15 lb/in^2 for 20 min and stored at 4°C.

- ## Solution II (freshly prepared):

0.2 N NaOH

1% SDS

Solution III:

5M Potassium Acetate	60 ml
Glacial Acetic Acid	11.5 ml
Milli Q	28.5 ml

The final solution autoclaved at 15 lb/in^2 for 20 min and stored at 4°C.

TBE buffer (5X): 1litre

Boric acid	27.5gm
Tris Base	54 gm
0.5M EDTA	20ml

Final volume maintained upto 1 liter, autoclaved at 15 lb/in^2 for 20 min and stored at 4°C.

6X Gel loading dye:

Bromophenol blue	0.03%(W/V)
Xylene cyanol FF	0.03%
Glycerol	60 %
60 mM EDTA	
10 mM Tris (pH-8.0)	

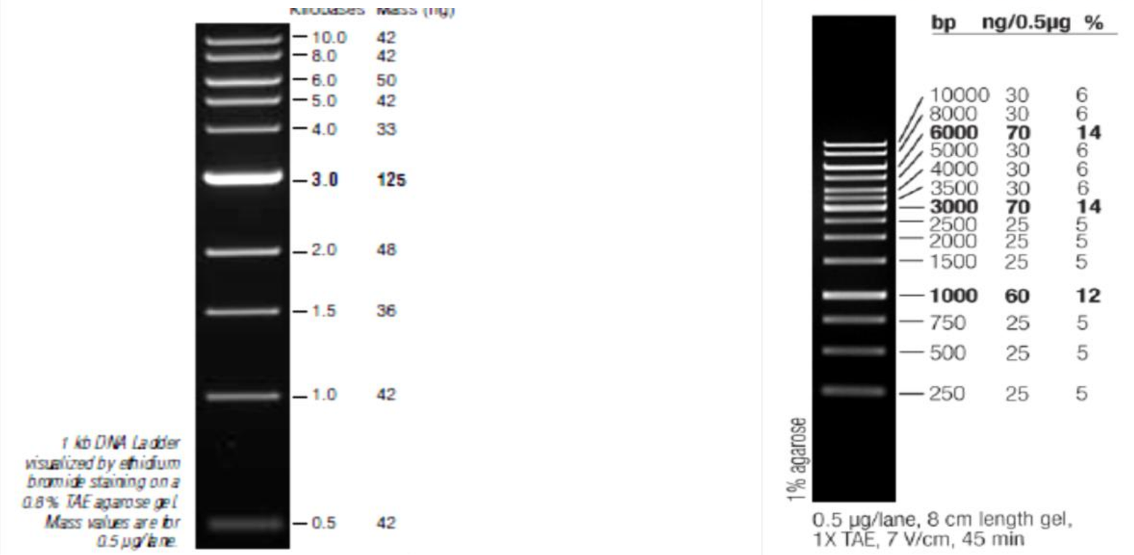
Immunofluorescence Microscopy Reagents (Fixative):

Paraformaldehyde	2 gm
0.5 M EGTA	100 µl
MiliQ	44.5 ml

The solution was heated (not boiled) and then cooled for 15 minutes and then 5 ml of 10X PBS and 125 µl of 1 M $MgCl_2$ were added to it.

- ## Quencher:

50mM NH_4Cl	0.27gm
PBS	100 ml



NEB 1 kb DNA Ladder

GeneRuler 1 kb DNA ladder (Fermentas)

Figure 8.1: Markers used for restriction analysis.