EXPRESSION AND LOCALIZATION OF A FLUORESCENTLY TAGGED t-SNARE SYNTAXIN 4 IN IMMUNE CELLS

M.Sc. Thesis

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CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by

Gauri Thapa

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Supervisors

Supervisor
Prof.Dr. Rajani Malla
TU
Co-Supervisor
Tarka Raj Bhatta

Research supervisor
Assistant Prof. Dr Niti Puri
School of Life science
JNU

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Gauri Thapa

List of Abbreviations

aa	amino acid
Ab	antibody
ATP	Adenosine triphosphate
bp	base pair
cfu	colony forming unit
DiIC	dialkyindocarbocyanine
DNA	Deoxyribo nucleic Acid
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetracetic acid
EGFP	Enhanced Green Fluorescence Protein
ECL	Enhanced chemiluminescence
FBS	Fetal Bovine Serum
Gm	Gram
HEPES	2-[4-(2-Hydroxyethly)-1-piperazine]ethane sulfonic acid
Hr	hour
IgE	Immunoglobulin E
Kan	Kanamycin
Kb	kilobase pair
LB	Luria Bertani
MCS	Multiple cloning site
mg	Milligram
ml	Milliliter
mM	Mill molar
Munc	Mammalian uncoordinated
ng	Nano gram

NSF	N-ethylmaleimide-Sensitive Factor
OD	Optical Density
PBS	Phosphate Buffer Saline
RBL	Rat Basophilic Leukemia
rpm	rotations per minute
RPMI	Rosewell Park Memorial Institute
RT	Room Temperature
SM	Sec1/ Munc 18
SNAP	Soluble N-ethylmaleidine-Sensitive Factor Attachment Protein
SNARE	Soluble N-ethylmaleidine-Sensitive Factor Attachment Protein Receptor
STX	Syntaxin
TBE	Tris Borate with EDTA
Tris	Tris(hydroxymethyl) aminoethane
t-SNARE	target SNARE
µl	Microlitre
µg	Microgram
VAMP	Vesicle Associated Membrane Protein
v-SNARE	vesicle SNARE

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Mast cells are fascinating, multifunctional, bone marrow derived, tissue-dwelling leukocytes that play a central role in immediate allergic reactions and also can be important as initiators and effectors of innate immunity and adaptive immunity. The trigger for the degranulations of different cytokines and chemokines have specific set of SNARE proteins that have not been fully studied. This study focuses on expression and localization of fluorescently tagged STX4 in mast cells and cloning of SNAP25 in pEGFP. Expression and localization of previously cloned t–SNARE STX4 in pEGFP and pDsRed was studied by using flow cytometry and immunoflourescence microscopy technique and cloning and expression of t-SNARE SNAP25 was done in pEGFP. Expression of STX4 in mast cell line model was observed by western blotting. Confirmation of previously cloned STX4 in pEGFP and pDsRed was done by restriction digestion. Both transfected plasmids into RBL cells showed transfection in flow cytometry. Immunoflourescence microscopy showed pEGFP STX4 localized on plasma membrane but STX4 in pDsREd could not get expressed due to frame shift while cloning. The localization of pEGFP STX4 on plasma membrane was observed clearly at 24 hours. Cloned pEGFP SNAP25 plasmid sequence showed one mutation in 451 position from A to G transition from CTA to CTG and overexpression of transfected RBL cells with pEGFP. SNAP25 localized on plasma membrane. However, the exact role of all these SNARE proteins involved in release of various mast cell mediators is still completely unknown. This study was designed to prepare tools for investigation of roles of t-SNAREs namely STX4 and SNAP25 in mast cell function by microscopic imaging studies in real time.

Keywords: Mast cell degranulation, SNAREs, pEGFP, PDsRed, STX4, SNAP25

CHAPTER 1 INTRODUCTION

1.1 Background

Membrane bound organelles are the important and distinguishing features of eukaryotic cells. Survivelity of the cells need transport system of molecules, proteins from one cell organelle to the other without disturbing the homeostatis of the cells. This transport system of molecules or intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell–cell contact or transfer of secreted molecules and also transfer of extracellular vesicles (Boyle, J. 2008 Molecular biology of the cell). It occurs intracellularly in membrane trafficking and exocytosis, including neurotransmitter release in synaptic transmission. Fusion between cellular membranes can occur constitutively, as seen when a transport vesicle from the endoplasmic reticulum fuses with the Golgi, or fusion can be closely regulated, as when a secretory storage vesicle fuses with the surface membrane upon receipt of a signal, a process termed as exocytosis (Pfeffer and Rothman, 1987). Its final step is the process of granule or vesicular fusion with the plasma membrane and is accompanied by release of granule/vesicle contents to the cell exterior. This transport between organelles or compartment in eukaryotic cells is accomplished by a series of membrane-bound carrier with vesicular SNAREs *(Soluble N-ethylmaleimide-sensitive factor attachment protein receptors)* that are loaded with soluble and membrane-bound cargo bud off a donor compartment, and move to, then dock and fuse with a target membrane SNAREs.

In 1987 Glick and Rothman reported golgi-associated protein factor (termed NSF) is required for transport between cisternae of the Golgi intracellular membrane fusion. Weidman *et al*., 1989 showed the existence soluble NSF attachment protein (SNAP) that together bind NSF to Golgi membranes. These SNAP protein family have three peripheral proteins also known as α-SNAP, β-SNAP and γ-SNAP proteins. Among them, only α-SNAP which is found in yeast as well as animals which take part in the membrane fusion machinery and has been conserved through evolution (Clary *et al*., 1990). SNAP protein binds to specific proteins in the membrane known as SNAREs and dissociates the complex SNARE motifs into unitary form. These SNAREs consists of VAMP 2 (Vesicle associated membrane protein) also known as vesicular SNAREs, syntaxin 1 and SNAP 25 combinely known as target SNAREs which forms a complex. This complex binds α-SNAP and then allows NSF to bind to it and brings membrane fusion in neuronal synapses (Sollner et al., 1993). This pairing of v-SNARE with t-SNARE along with accessory proteins is known as " The SNARE hypothesis". Botulinum toxins and tetanus toxin are potent inhibitors of synaptic vesicle exocytosis in neuronal synapses (Link *et al*., 1992). The SNARE proteins are present in free form inside the cell which is prone to cleavage by toxins before they form a ternary complex (T.Hayashi *et al*., 1994). This ternary complex is also known as SNARE pin which is only formed between a cognate v-SNARE and t-SNARE (Weber *et al*., 1998).The spin labeling electron paramagnetic resonance spectroscopy of the SNARE complex gave us a helical budle of four parallel strands consisting of VAMP, syntaxin and two parallel strands of SNAP 25 together which is palmolyted to the plasma membrane (Poirier *et al*., 1998). The SNARE

machinery of membrane fusion involves different sets of proteins of vesicle and target SNARE that lie on opposing membranes. Their subsequent zippering into stable membrane-bridging (''trans'') complexes would bring the membranes gradually into close apposition, eventually leading to membrane merger (Hanson *et al*., 1997). The fusion is formed with a high stable tetrameric trans-SNARE complex through four conserved 60–70 aa SNARE motifs. After fusion, SNARE complexes are transformed from a trans- to cis- configuration (Sutton *et al*., 1998). Parlati *et al*., 2002 tested 275 distinct quaternary SNARE combinatsions with combinatorial specificity that has fusion potential. With the introduction of new SNARE motifs they had characteristics of both vesicle as well as target SNARE. Therefore, the SNARE proteins were segregated into four major subfamilies based on the amino acid sequence homologies of the SNARE domains. The classification of Qa (syntaxin), Qb, Qc (SNAP 25/23 residues) where Q had glutamine residues and R (VAMP) had arginine residue SNAREs is based on the residue at the position of the zero ionic layer of the 4-helical bundle (Hong ., 2005).

Mast cells are leukocytes that are derived from hematopoietic progenitor cells that has evolutionarily conserved functions in pathogen surveillance.They are dispersed throughout most tissues but are crucially located at the host's interfaces with the environment, such as the skin and mucosae, supporting a role in the recognition of pathogens or other signs of infection (Kindt, T. J, Kuby, J. 2007. Kuby immunology). Mast cells had been most intensively studied in the context of allergic disease, such as allergic asthma and rhinitis. However, over recent years, the contribution of mast cells to many other aspects of host defense has become recognized (Abharam and John ., 2010, Urb and Sheppard ., 2011). Mast cells can function as effector cells during innate and adaptive immune responses. In 'effector' functions of mast cells include killing pathogens degrading potentially toxic endogenous peptides or components of venoms and regulating the number, viability, distribution, phenotype and 'non-immune' functions of structural cells, such as fibroblasts and vascular endothelial cells. Mast cells can exert their effector functions through the direct or indirect actions of a wide variety of mast-cell-derived products, and such functions can be observed in both innate and adaptive immune responses (Galli *et al*., 2008). Armed with granules containing preformed mediators, mast cells have the potential to be the first responders (within seconds to minutes) following recognition of an invading pathogen. Upon activation of the cell, the mediators are released via a vesicle fusion mechanism with the plasma membrane. Fusion can be activated through receptor stimulation or by membrane depolarization via 2^{nd} messengers, for example Ca^{2+} (Jahn *et al*., 2004). They use a highly sophisticated machinery of proteins that enable the fusion of vesicles containing pre-stored and newly synthesized inflammatory mediators (Lorentz *et al*., 2012). Mast cells express a wide array of SNAREs. SNARE proteins in mast cells include the t-SNAREs SNAP-23 as well as STX2, 3, 4, and 6. VAMP family protein members include VAMP2, 3, 4, 7, and 8 (Sander *et al*., 2008, Benhamou and Blank, 2010). But, the exact role of all these in exact fusion steps involved in release of various mast cell mediators is still completely known. This study was designed to prepare tools for investigation of roles of t-SNAREs namely STX 4 and SNAP 25 in mast cell function by microscopic imaging studies in real time.

1.2 Research Plan

1.2.1 Research Plan for localization of Syntaxin 4

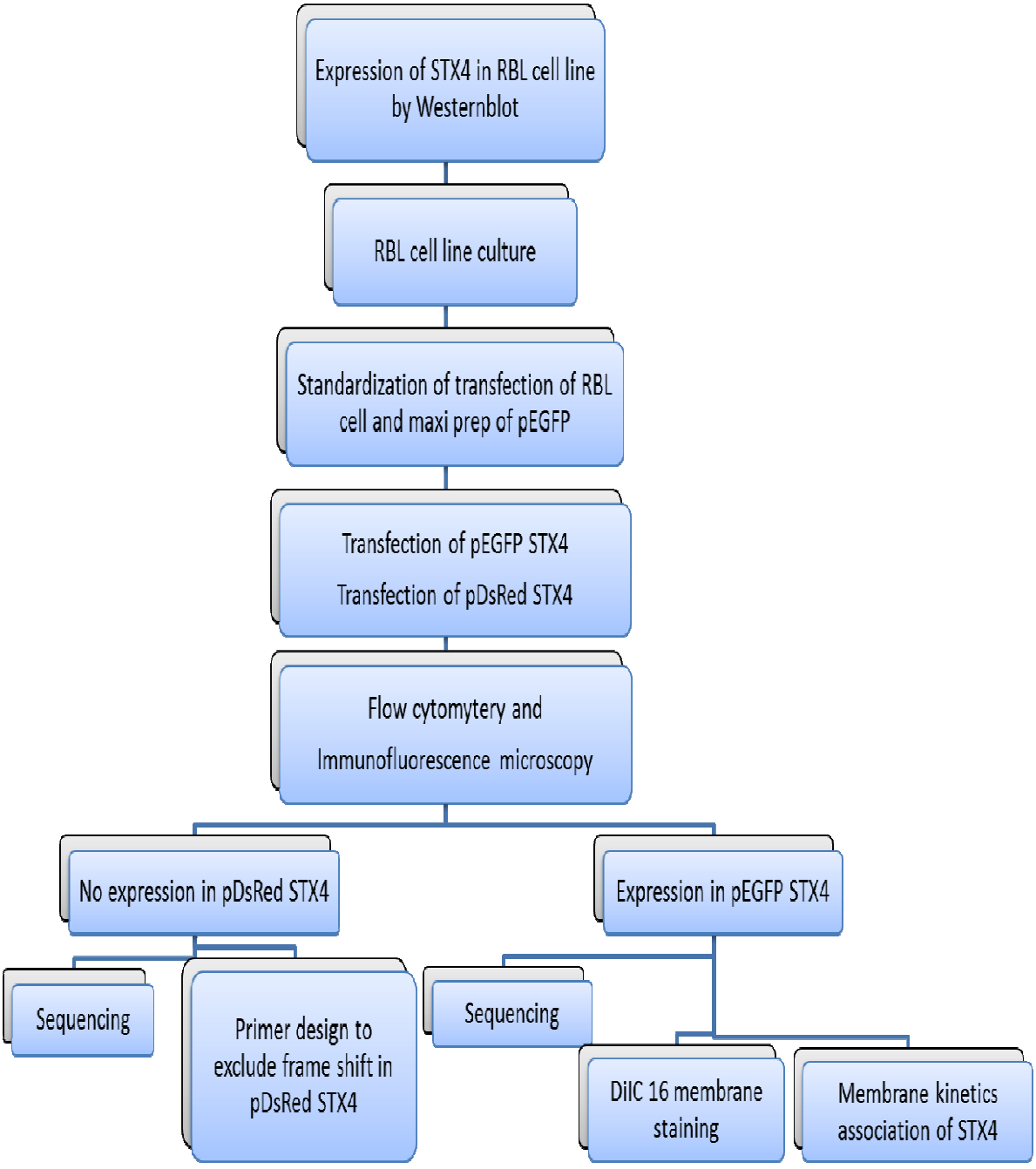


Figure 1.1: Flow diagram showing Syntaxin 4

1.2.2 Research plan for cloning of SNAP 25

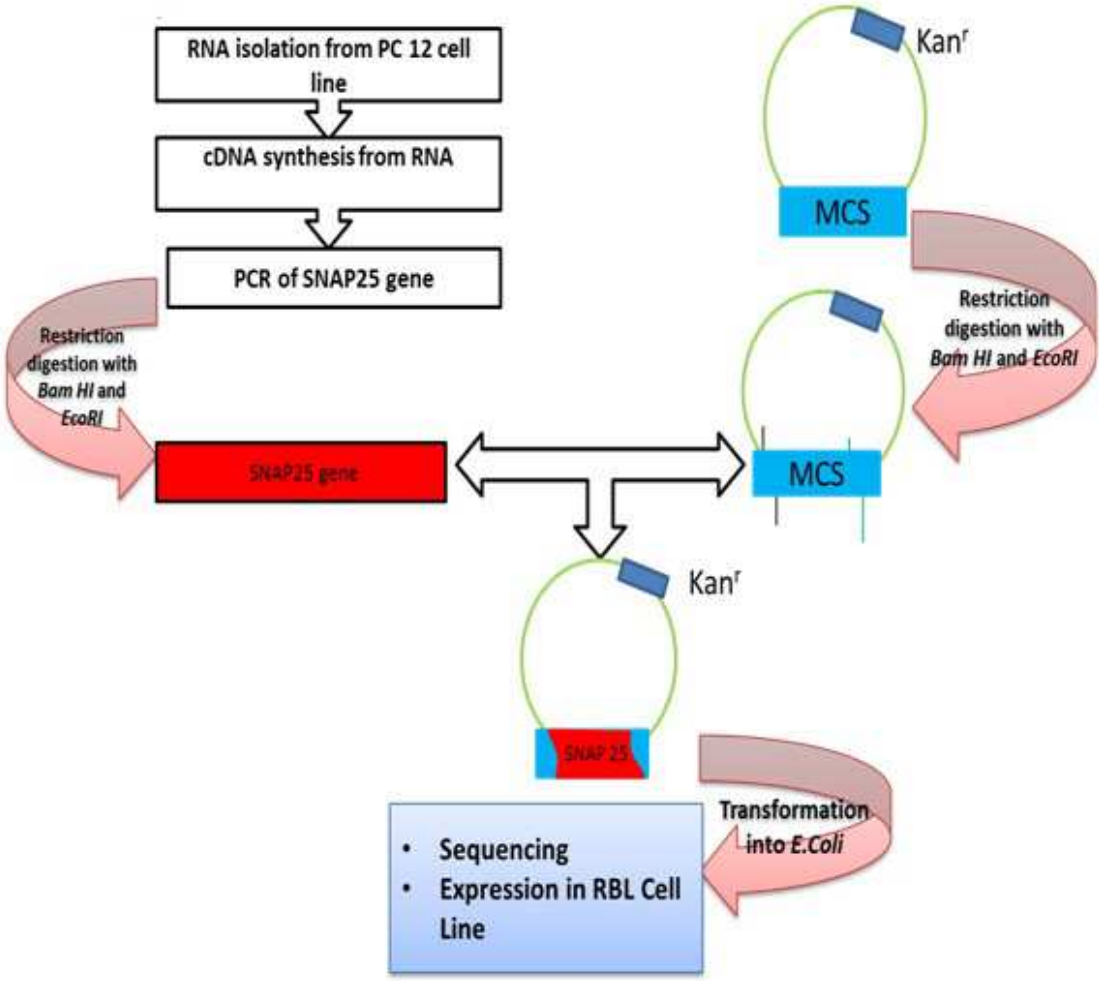


Figure 1.2: Flow diagram showing cloning of SNAP 25

1.3 Objective

General Objective

To study the expression and localization of fluorescently tagged syntaxin 4 in mast cells and cloning of SNAP25 in pEGFP vector.

Specific Objective

- To study the expression of syntaxin 4 in RBL cell line,
- To standardize transfection in RBL cell line,
- To confirm previously cloned pEGFP STX 4 and pDsRed STX 4,
- To study membrane kinetics association of pEGFP STX 4 in RBL cell line,
- To study the co-localization of syntaxin 4 with plasma membrane,
- To clone SNAP 25 from PC 12 cell line and check its expression in RBL cell line

1.4 Rationale

Mast cells also requires a specific set of proteins called *Soluble N-ethylmaleimide-sensitive factor attachment protein receptors* (SNAREs) that are highly conserved in all eukaryotes (Sudhof *et al* 2011). The studies of fusion protein like SNAREs have great therapeutic potentials in mast cells. Recent studies have shown that T20 (Enfuvirtide, Fuzeon), a 36-amino acid peptide derived from the C-terminal heptad repeat region of HIV-1 gp41, is the first and only clinically approved HIV-1 fusion inhibitor that being used for treatment of HIV/AIDS patients failed to respond to current antiretroviral drugs as fusion of viral and cellular membranes is an essential step for HIV-1 infection and this process is an attractive target for developing antiviral agents (He *et al., 2*013). By studying the proteins involved in the secretion of different mediators we can either up regulate it or down regulate the secretion of different mediators the help of different regulators and inhibitors. As mast cell degranulation can cause detrimental effects in diseases like Abdominal aortic aneurysm formation, asthma, lung fibrosis (bleomycin) and arthritis (Woska *et al* ,2012) we can use the study of SNAREs and its interaction with different compounds as a model for therapy of various diseases.

1.5 Research Hypothesis

Null hypothesis (H_o): Previously cloned plasmids pEGFP STX 4 and pDsRed STX 4 does not gets expressed and localized in plasma membrane of RBL cells and SNAP 25 gene cloned in pEGFP vector form PC 12 cell line does not gets overexpressed in RBL cells.

Alternate hypothesis (H_1): Previously cloned plasmids pEGFP STX 4 and pDsRed STX 4 gets expressed and localized in plasma membrane of RBL cells and SNAP 25 gene cloned in pEGFP vector form PC 12 cell line gets expressed in RBL cells.

CHAPTER 2 LITERATURE REVIEW

2.1 SNAREs mediate membrane fusion and thereby vesicle traffic/protein traffic

Eukaryotic cells contain membrane-enclosed organelles that communicate with each other through the exchange of trafficking vesicles. Trafficking usually involves the generation a vesicle from a precursor membrane, the transport of the vesicle to its destination and, last, the fusion of the vesicle with the target compartment (Juan *et al*., 2004). At the synapse a promenade of protein-protein interactions mediate the docking, priming, and fusion of synaptic vesicles (Bock *et al*., 2001). Central to the process of membrane fusion are SNARE proteins, which are localized to various intracellular organelles and membranes in all eukaryotic cells (Jahn and Scheller, 2006,Martens and McMahon, 2008,Szule *et al., 2*003), and also play a well-known role in neurotransmitter release from presynaptic terminals (Sollner *et al., 1*993).SNAREs form clusters which contain cholesterol in the plasma membrane that define sites at which secretory vesicles dock and fuse with high preference(Lang *et al., 2*001). Fusion of exocytic vesicles that have been activated and attached to the plasma membrane is triggered by Ca2+ influx into the cytosol (Mayer *et al*., 2001).

The SNARE family of evolutionarily conserved proteins was first identified in the 1980s in yeast and a decade later in mammalian cells. SNAREs are found in most eukaryotic cells; 25 members have been identified in Saccharomyces cerevisiae, 54 members in Arabidopsis thaliana and 36 members in humans (Woska *et al*., 2012). SNARE hypothesis for the targeting of transport vesicles which basically, comprise three highly conserved protein families, each each including several members (Rothman *et al., 1*994) postulates that each kind of transport vesicle is endowed with its own, unique vesicle- (v-) SNARE that forms a unique match with its cognate target- (t-) SNARE, the latter being present only at the intended target membrane. In general, the v-SNAREs would be expected to consist of molecules related to VAMP in sequence and/or structure, whereas t-SNAREs would be related to and would probably function as hetero-oligomers shown in figure (Rothman *et al., 2*004). Two steps of membrane attachment can be distinguished: tethering and docking. In cooperation with large, organelle-specific complexes of tethering factors, Rab-like GTPases mediate tethering, an initial binding of the fusion partners (Mayer *et al*) (Rothman *et al., 1*994). SNARE complex holds these two parts together. The SNARE complex would then have to form at some point before the fusion pore opens and neurotransmitters are released (Han *et al., 2*004). SNAREs form clusters in the plasma membrane that defines sites which secretory vesicles dock and fuse with high preference. The clusters contain cholesterol and removal of these cholesterol cause dispersion of these clusters which is associated with inhibition of exocytosis (Lng *et al*., 2001). The study of SNARE proteins have been due to the action of many toxin proteins which act on different SNARE proteins like, VAMP/synaptobrevin isoforms are cleaved by tetanus toxin (TeTx) and botulinum toxin (BoTx) B, D, F and G, SNAP-25 isoforms by BoTx A, C and E, and Syntaxins by BoTx C (Schiavo *et al* 2000) however VAMP-7 and VAMP-8 however are insensitive to the proteolytic activity of neurotoxins (Sander *et al., 2*008).

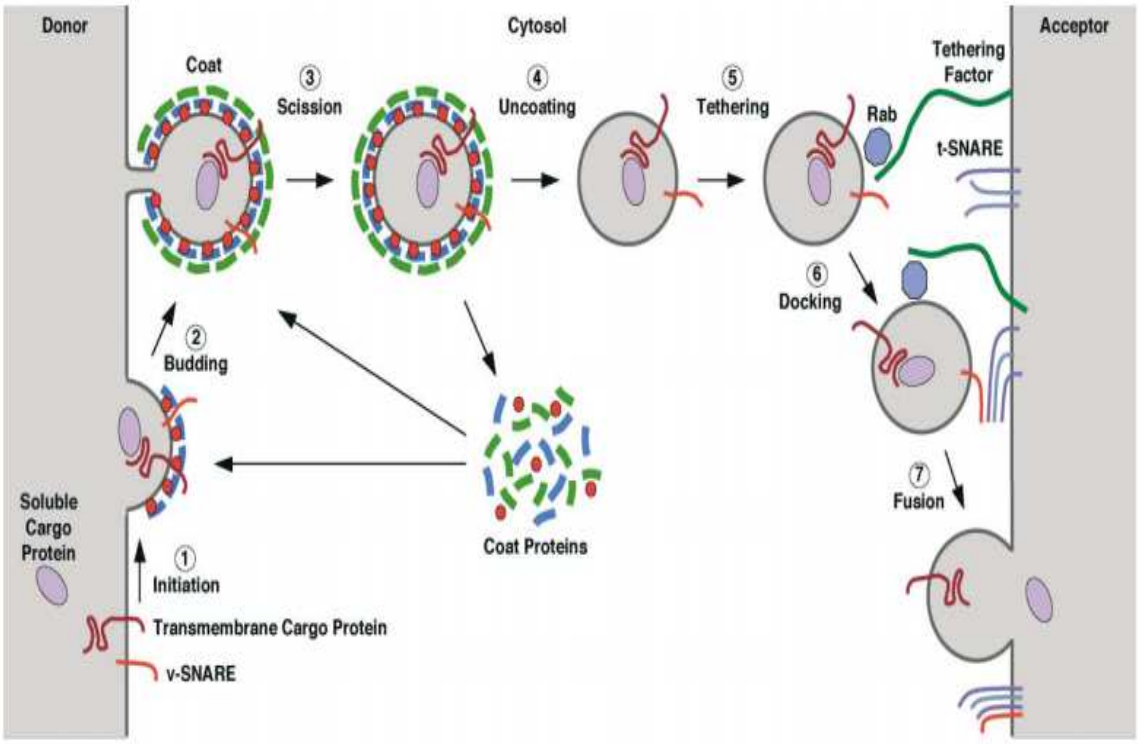


Figure 2.1: Steps of Vesicle Budding and Fusion:

(1) Initiation of coat assembly: The membrane-proximal coat components (blue) are recruited to the donor compartment **(2) Budding:** The membrane-distal coat components (green) are added and polymerize into a mesh-like structure. **(3) Scission:** The neck between the vesicle and the donor compartment is severed either by direct action of the coat or by accessory proteins. **(4) Uncoating:** The vesicle loses its coat due to various events including inactivation of the small GTPase, phosphoinositide hydrolysis, and the action of uncoating enzymes. **(5) Tethering:** The "naked" vesicle moves to the acceptor compartment, possibly guided by the cytoskeleton, and becomes tethered to the acceptor compartment by the combination of a GTP bound Rab and a tethering factor. **(6) Docking:** The v- and t-SNAREs assemble into a four-helix bundle. **(7) This "*trans*-SNARE complex"** promotes fusion of the vesicle and acceptor lipid bilayers. Cargo is transferred to the acceptor compartment, and the SNAREs are recycled (Juan *et al*., 2004).

SNAREs are highly abundant for example, syntaxin-1 and SNAP-25 each constitute ~1% of the total brain protein important SNARE regulatory molecules include SNAP and NSF, which catalyse SNARE complex disassembly following membrane fusion (Salau *et al*., 2004). SNARE proteins are essential in several immunohematological cells, including neutrophils, mast cells, macrophages, cytotoxic T cells, platelets, and NK cells. SNARE molecular machinery controls the constitutive antibody secretion by human plasma cells. In mast cells, the t-SNARE component SNAP23 is relocated to granule membranes to allow for membrane fusion during compound exocytosis increased SNAREs allow for increased vesicular traffic and secretion of cytokines in activated macrophages (Pagan *et al*., 2003).

SNAREs molecules regulate cardiac myocyte exocytosis of ANP: VAMP-1, and VAMP-2, partially co-localize with ANP granules and syntaxin-4 modulate exocytosis of ANP from cardiac myocytes (Ferlito *et al., 2*010). SNARE-mediated fusion midbody abscission in mammalian cells, a highly regulated, active process in mammalian cells accomplish the breakage of this narrow bridge which causes cells "rip apart "or "pinch off" to complete cytokinesis (Low *et al*., 2003).

2.2 Structure of SNAREs

SNARE complexes are rods, 13–14 nm long and approximately 2 nm wide coiled-coil structure (Weber *et al*., 1998). Most SNAREs are C-terminally anchored trans membrane proteins, with their functional N-terminal domains facing the cytosol. Each of these proteins contains a heptad repeat "SNARE motif" of 60–70 amino acids that can participate in coiled-coil formation (Bock *et al*., 2001). An exception is SNAP-25, which contains two SNARE motifs and binds to the membrane via covalently linked palmitate groups attached to the central part of the protein (Juan *et al.,* 2004).

Based on the amino acid in the ionic layer (glutamine or arginine) and other conserved structural motifs, SNAREs can be further subdivided into four sub-families: syntaxin/Qa-, Qb, Qc- and RSNAREs. Consistent with this sequence based classification, a fusogenic SNARE pin contains always one member of each of the four SNARE sub-families (Sollner *et al., 2*003). The crystal structures of two, only distantly related, SNARE core complexes have revealed a remarkable degree of conservation. Core complexes are represented by elongated coiled coils of four intertwined, parallel α-helices, with each helix being provided by a different SNARE motif (figure 4). The centre of the bundle contains 16 stacked layers of interacting side chains. These layers are largely hydrophobic, except for a central '0' layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residues shown in fig .According to the functional residue amino acid in the conserved SNARE motif SNARE motifs are classified into Qa-, Qb-, Qc- and R-SNAREs. Functional SNARE complexes that drive membrane fusion are hetero-oligomer, parallel four-helix bundles, and each bundle is invariant, requiring one of each of the Qa-, Qb-, Qc- and R-SNAREs (Hang *et al*., 2005).

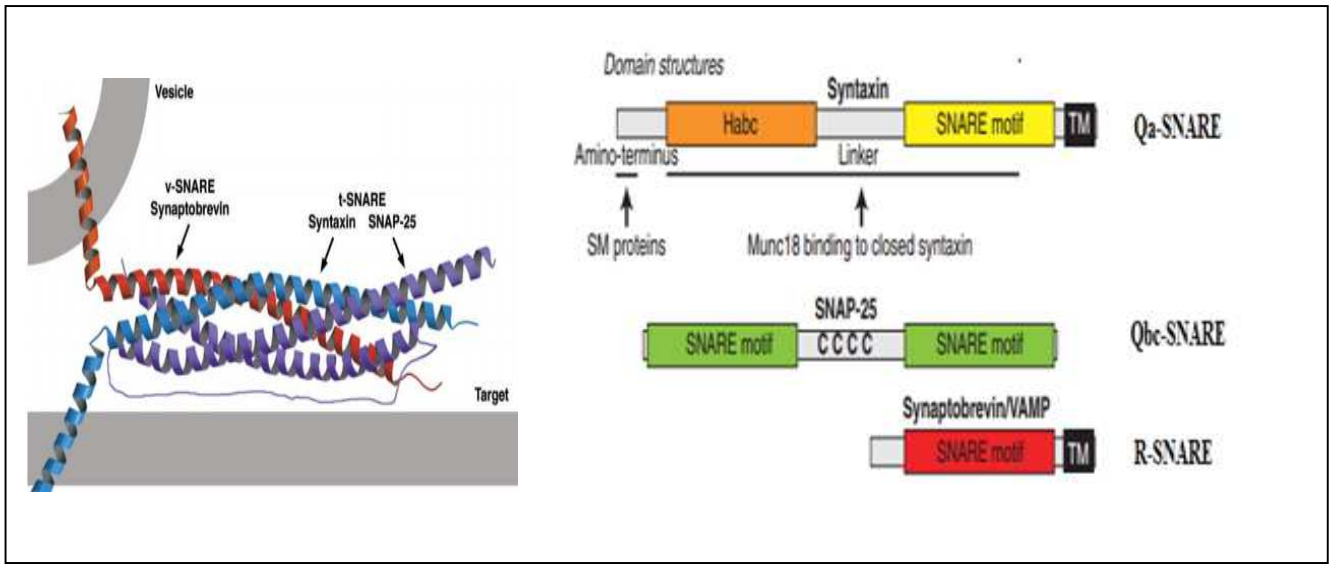


Figure 2.2: Model of SNARE fusion complex:Core SNARE complex formed by four α-helices contributed by synaptobrevin, syntaxin and SNAP 25, Structure of SNAREs: Different SNARE proteins with their SNARE motifs and binding region of regulatory proteins (Sutton et al)

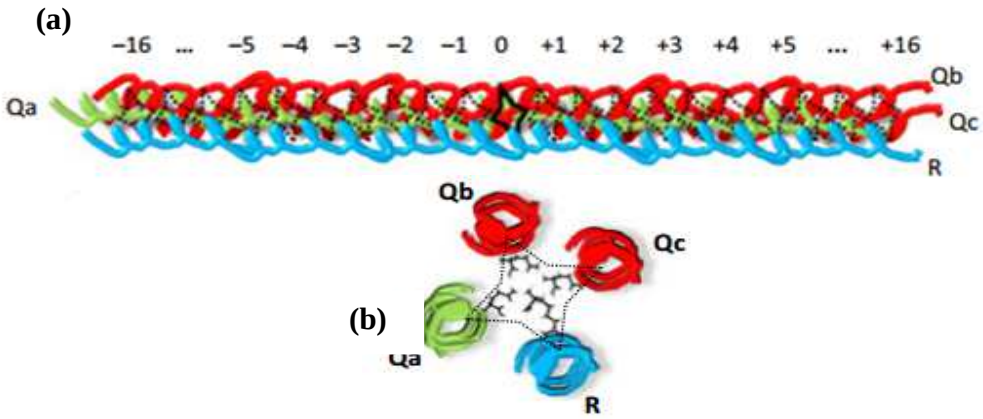


Figure 2.3: Structure of SNARE core complex

(a) Four SNARE motifs from each of the Qa, Qb, Qc and R families form a functional four helical bundle, which drives membrane fusion. **(b)** The QabcR configuration of the zero layers.

2.3 SNARE in vesicle trafficking

SNARE proteins are localized in opposing membranes which interact to form a highly stable 'SNARE complex 'and involves the interaction of coiled-coil (helical) domains present in the individual SNARE. It forms a parallel, twisted four-helix bundle bundles in which the four SNARE motifs are in a parallel orientation, with the trans membrane regions emerging from C-terminal end (Jahn *et al*., 2003). They drive membrane fusion by using the free energy which is released during the formation of a four helix bundle (Suzie *et al., 2*008) (Salau *et al*., 2004) (Rothman *et al*., 1994) (Sollner *et al*., 1993).

When SNAREs are monomeric, SNARE motifs are unstructured. However, when appropriate sets of SNAREs are combined, the SNARE motifs spontaneously associate to form helical core complexes of extraordinary stability. SNARE complex assembly involves the interaction of coiled-coil (helical) domains present in the individual SNARE proteins to form a parallel, twisted four-helix bundle. N- to C-terminal zipping and the associated conformational change in the C terminus of the SNARE complex is thought to induce fusion pore formation (Sorensen *et al*., 2006). Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. This fully zipped state is thought to lead to fusion pore opening the C terminus of the four-helical bundle will be zipped less tightly, which should lead to a longer fusion pore it is possible that synaptotagmin affects the fusion pore mechanics. It is also possible that the final zipping of the SNARE complex is not producing a unique structure but might depend on specific Ca2/synaptotagmin action, leading to variability in the conformation of SNARE complexes that lead to fusion. The transition from the primed state to the fully zipped state is triggered by Ca2 via a mechanism involving synaptotagmin and complexin links of the SNARE domains to the trans membrane domains of synaptobrevin and syntaxin and their interactions with the membranes, the tension exerted by this fully zipped complex relaxes via opening of the fusion pore (Fang *et al., 2*008). The recycling of SNAREs is achieved through the dissociation of the helical bundle, which is mediated by ATP-driven chaperone NSF (mammalian ortholog of yeast Sec18p) and its cofactor α-SNAP (mammalian ortholog of yeast Sec17p), yielding activated SNAREs that are then competent to reform trans-complexes associations between Q- and R-SNAREs from opposing membranes. The ability of NSF to untwist and dissociate SNAREs is called SNARE priming (Klrnchin *et al* 2000) and Puri *et al*., 2003 has shown that mutant of NSF acts as an inhibitor of SNARE disassembly in vitro.

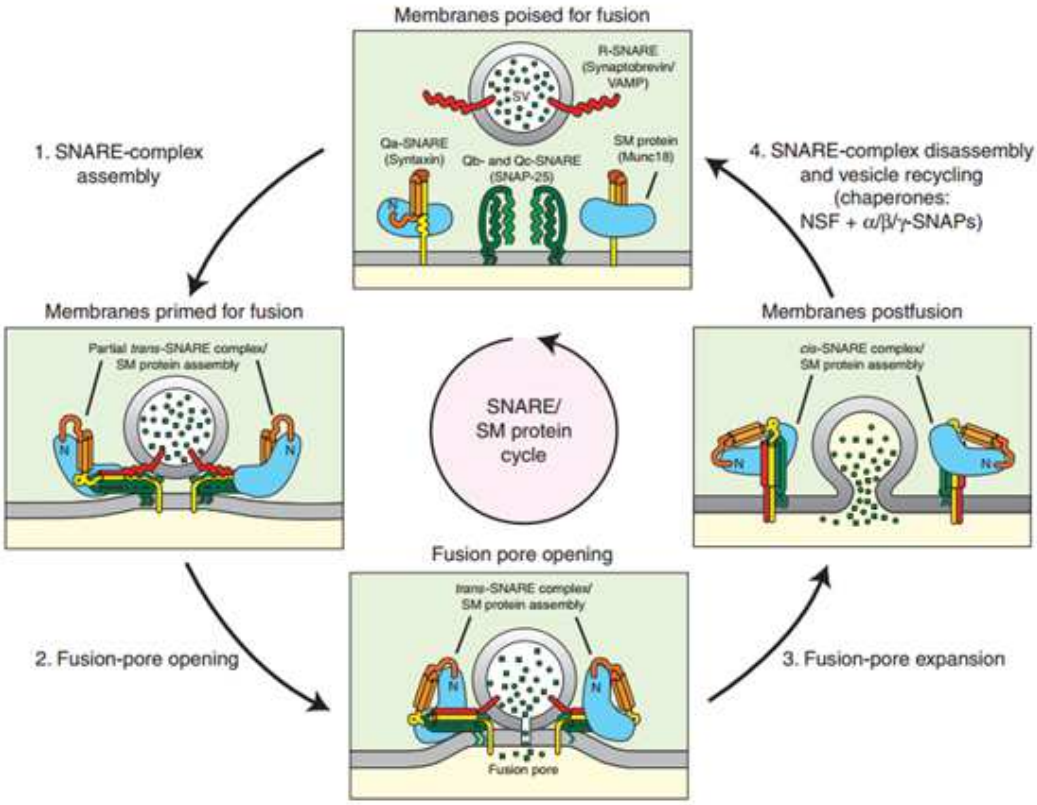


Figure 2.4: The SNARE/SM protein cycle

The diagram on top depicts SNAREs prior to fusion, when they are localized to the membranes.During priming, SNARE proteins partially zipper up into trans-complexes. Full SNARE-complex assembly then pulls the membranes apart, opening the fusion pore, then, trans-SNARE complexes are converted into cis-SNARE complexes. Afterward, cis-SNARE complexes are dissociated by the ATPase NSF adaptors and α-SNAPs, and vesicles recycle to start another round of the cycle (Sudhof *et al., 2*013)

2.3.1 Qa SNARE

Members of the syntaxin family of SNARE proteins are found on numerous intracellular organelles, and their function is required for a wide range of intracellular membrane fusion pathways. Syntaxins belong to a family of proteins that are 'tail-anchored' (also called Type IV membrane proteins); such proteins have an NH2 terminal cytoplasmic domain that is membrane-bound by virtue of a single C-terminal hydrophobic domain and have no ectodomain. In mammalian cells, four of the 15 members of the syntaxin family, STX1-4, localize to the plasma membrane (PM), where they form small (50-60 nm) homotypic clusters of approximately 70 molecules that are thought to mark sites of exocytosis on the cell surface. all syntaxins have a regulatory domains , the Habc domain an amino-terminal three-helix bundle t-SNARE light chain Vtib (Sollner *et al.,* 2003). Syntaxins 2, 3 and 4 are cell surface proteins, syntaxin 5 and 6 are localized to the Golgi region and syntaxin 7 is localized to the

endosome. Syntaxin 10 is localized to the trans-Golgi network, syntaxin 12 which appears to be endosomal and syntaxin 16, whose myc tagged form is localized to the Golgi apparatus (Teng *et al*., 1998).

Syntaxin 1 (which is expressed almost exclusively in neuronal and neuroendocrine cells) functions in exocytosis pathways such as presynaptic neurotransmitter release, whereas syntaxin 4, which has a more ubiquitous tissue distribution, functions in pathways such as the exocytosis of vesicles containing the facilitative glucose transporter, Glut4, in adipocytes and skeletal muscle.

Syntaxin 1A exists in at least two distinct conformations: in an open conformation, syntaxin 1A is able to form functional SNARE complexes, whereas munc18-1 holds syntaxin 1A in a SNARE complex-incompatible 'closed' conformation (Salaun *et al*., 2004). Wei Li *et al*., 2011 has shown that syntaxin 1A is able to form functional SNARE complexes, whereas munc18-1 holds syntaxin 1A in a SNARE complex-incompatible 'closed 'conformation. Syntaxin-1 SNARE motif, and likely to Munc18-1, helps to ''extract'' the SNARE motif from the closed conformation, thus allowing Syntaptobrevin and SNAP-25 binding to form the SNARE complex.

Syn-1A, a SNARE protein known to mediate membrane fusion, is not only found in non-secretory cardiac ventricular sarcolemma, but also inhibits acid- and KATP channel opener-induced KATP channel activation through increased binding and interaction with SUR2A-NBF-1 and NBF-2 (sulfonylurea receptor (SUR) 2A nucleotide binding folds NBF) (Chao *et al.,* 2011). Syntaxin 6 is distinct from previously characterized proteins implicated in vesicle trafficking, due to its similarity to both the syntaxin and SNAP-25 families syntaxin 6 may serve a function which requires both SNAP-25 and syntaxin 1 at the Golgi to plasma membrane step (Bock *et al., 1*996) syntaxin 2 and 4 are required for lysosome release (Chen *et al., 2*009).

It was later stated in oh *et al*'s study that Syntaxin 1 regulates only first-phase insulin secretion, and Syntaxin 4 facilitates both first and second phases. Syntaxin 4 was limiting for peak insulin release from healthy human islets, and its enrichment enhances glucose-regulated biphasic insulin release by 100% (Oh *et al*., 2014). STX4 enhances or inhibits myogenic differentiation via regulation of promyogenic signalling molecules Cdo and p38. STX4 and Cdo interact physically in differentiating myoblasts, and this interaction is mediated by the t-SNARE domain of STX4, regulates translocation of Cdo to the plasma membrane (Yoo *et al*., 2015). Syntaxin 4 in particular is a Qa SNARE that has been shown to play an important role in regulated secretion on the plasma membrane of neutrophils, macrophage ,eosinophil's and also STX4 is necessarily involved in the secretion process of Ig by human PCs (Rahman *et al., 2*013, Murray *et al., 2*006, Jaramillo *et al*., 2013).

Syntaxin 4 in RBL cells not only interacts with SNAP-23, another t-SNARE, but also with several v-SNAREs including VAMP-2, -3 and -8. STX4 defines exocytic zone for activity-dependent spine modification required for synaptic plasticity. STX4 localizes to dendritic spines where it marks sites where spine endosomes fuse with the spine PM, thus enabling synapse-specific membrane delivery (Kennedy *et al*., 2014).

Syntaxin-4 and not VAMP3 plays a vital role in exocytosis of IgE from plasma cells. Syntaxin-4 co-localises with IgE in plasma cells in U266 multiple myeloma plasma cell line (Rahman *et al.,* 2013). Syntaxin-1 SNARE motif, and likely to Munc18-1, helps to 'extract' the SNARE motif from the closed conformation, thus allowing Syntaptobrevin and SNAP-25 binding to form the SNARE complex.

2.3.2 Qb, Qc SNARE

The synaptosome associated protein (SNAP) family of SNAREs is an example of the Qbc subtype and contain two SNARE motifs joined by a flexible linker and that is palmitoylated and therefore lacks a trans membrane domain. Members of the SNAP-25 protein family contribute two of the four alpha helices that compose exocytic SNARE complexes. Alpha helical SNARE motifs are present at the N- and C-termini of SNAP-25 proteins and are separated by a central cysteine-rich membrane targeting/binding domain The first identified SNAP-25 proteins were termed SNAP- 25A and SNAP-25B; It is mainly found in neuronal cells which do not contain TMDs where it is associated with the plasma membrane via plamitoylation on cysteine residues at the centre of the protein .Association of SNAP-25 and SNAP-23 with membranes is dependent upon a central cysteine-rich domain; this domain is palmitoylated in vivo and mutants lacking these cysteine residues are cytosolic, whereas SNAP-23 contains five cysteines in the cysteine-rich domain palmitoylation (Puri *et al., 2*004). The minimal domain of SNAP-25 required for efficient palmitoylation is a 36-amino-acid sequence containing the cysteine-rich domain and the 28 amino acids that follow the cysteines.SNAP-23 is 59% identical and 72% similar to SNAP-25 at the amino acid level, considering conservative amino acid substitutions. The regions of highest homology to SNAP-25 reside in the amino- and carboxyl-terminal thirds of the SNAP-23 protein there are four cysteine residues in both proteins clustered around SNAP-23 residue SNAP-23 is also capable of binding to different VAMP isoforms.

SNAP-23 in RBL-2H3 cells, co- precipitates with Syntaxin 4 and VAMP-8; however, NEM treatment was needed to observe VAMP-8 association in human mast cells isolated from surgical tissues (Sander *et al*., 2008 and Shukla *et al.,* 2000).

2.3.3 R-SNARE

VAMP (synaptobrevin)-family currently has eight described members: VAMP-1±8. VAMP-1 and VAMP-2 mainly exist in the brain, whereas the other VAMP-isoforms are ubiquitously expressed (Shukla *et al*., 2000). VAMPs are integral membrane proteins being anchored to the membrane of secretory granules by a trans membrane region adjacent to the C-terminus. VAMP-3 and VAMP-8 are required for platelet secretion (Polgar *et al., 2*002). VAMP8 is important for regulated exocytosis of salivary glands, lacrimal glands, in pancreatic acinar cells (Wang *et al*., 2004) and reasonable to speculate that VAMP8 is likely a common v-SNARE responsible for regulated exocytosis in the entire exocrine system (Wang *et al*., 2007).

2.4 SNARE regulators

Rab proteins comprise the largest family within the Ras superfamily of small GTPases. Individual Rab proteins are localized to distinct, characteristic organelles, suggesting that each regulates a

particular membrane traffic step. Rabs are thought to act as molecular switches, with the time spent in the active GTP-bound "on" state determining how long vesicles remain competent for docking/fusion, before GTP hydrolysis switches this state off (Rybin *et al.,* 1996).Sec4 effector in exocytosis (Rab1 effector in Golgi transport) (Terbush., 1997).Rab3 exists as four isoforms A-D in mammals and is the major Rab implicated in regulated exocytosis in neurons and neuroendocrine cell. Overexpression of active forms of Rab3A, or other Rab3 isoforms, has been found to inhibit exocytosis in chromaffin cells, PC12 cells, rat basophilic leukemia (RBL) cells , insulin secreting cells , pancreatic exocrine cell. The general consensus is that rabs are major determinants of the compartmental specificity of membrane transport and achieve this by selective interactions with distinct effector proteins (Holz *et al*., 1994 and Johhannes *et al.,* 1994). Overexpression of the granular-localized Rab3D isoform or expression of a preferentially GTP-bound mutant form (N135I) in RBL cells leads to a marked inhibition of degranulation (Roa *et al*., 1997, Tuvim *et al*., 1999). The best-characterized Rab effectors are involved in the initial tethering of vesicles to target membranes. Three family members are *mammalian uncoordinated18* (Munc18) proteins that are more specifically implicated in regulated exocytosis. They include Munc18-1 sometimes also called Munc18a; which is largely expressed in neurons and two ubiquitously expressed isoforms Munc18-2 (Munc18b) and Munc18-3 (Munc18c). Munc18-1 can interact with STX1, 2, and 3, Munc18-2 with STX1, 3, and slightly with STX2 Munc18-3 interacts with STX2 and 4 and to a lesser extent with STX1 and recently, Munc18-2 was also shown to interact with STX11In neutrophils, Munc18-2 could act as a regulator of primary granule exocytosis, while Munc18-3 may preferentially regulate the fusion of secondary/tertiary granules. , munc18-1 may facilitate the correct intracellular trafficking of syntaxin 1A by stabilizing it in a protected conformation that prevents its participation in unfavourable SNARE complexes (Pie *et al., 2*009, Higashio *et al.,* 2008).

Synaptotagmins are calcium-binding proteins containing two conserved binding domains at their C-terminus, C2A and C2B, and a single N-terminal transmembrane domain anchored to membranes of secretory vesicles (Sudhof *et al*., 2004). The binding of calcium to the C2 domains alters their electrostatic surface charge and mediates most of the calcium-dependent functions of synaptotagmin (Chapman *et al*., 2008). It is currently unknown which of the two membranes is the target of the synaptotagmin Ca2+-binding loops, but it is plausible that different orientations of the SNARE four helix bundle may position synaptotagmin toward the vesicle or plasma membrane, and insertion of the Ca2+-binding loops of different synaptotagmin molecules into both membranes may cause membrane perturbations that facilitate membrane fusion (Rizo *et al*., 2010).

2.5 Introduction to Mast cells

Mast cells were first described by Ehrlich in 1878. Mast cells are fascinating, multifunctional, bone marrow derived, tissue dwelling cells. They play a central role in inflammatory and immediate allergic reactions. There are two types of human mast cell, defined by relative tryptase and chymase content, also vary with respect to their expression of the receptor for complement component C5a. Activation of mast cells by pathogens can result in both degranulation and *de novo* cytokine synthesis. They can be activated to degranulate in minutes, not only by IgE and antigen signalling via the high-affinity receptor for IgE, but also by a diverse

group of stimuli. They are able to release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid that act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells. Mast cells arise in the bone marrow where maturation is influenced by stem cell factor binding to the receptor c-kit and by other cytokines such as interleukin (IL)-3, IL-4, IL-9, and IL-10(Amin *et al*., 2011). They circulate in the blood in an immature form before migrating to vascularised tissues, where they undergo final differentiation and maturation (Abharam *et al*., 2010).

Mast cells arise from bone marrow-derived precursors that circulate in the blood and become differentiated after entering tissues they are mostly found in locations that are in close contact with the external environment, such as skin, airways, and intestines. Activation of mast cells results in the release of a variety of soluble factors. They are long-lived cells, able to survive for months or years and, despite being terminally differentiated, they can proliferate in response to appropriate signals. Within seconds of stimulation, mast cells can undergo degranulation, rapidly releasing pre-formed mediators present within cytoplasmic granules, including histamine, the proteases tryptase and chymase, and pre-formed tumour necrosis factor α. Mast cells recognize pathogen invasion through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are activated in response to conserved pathogen-associated molecular patterns (PAMPs) they also detect range of products through the expression of other receptors that sense pathogens like , Fc receptors (FcRs) receptors, Nod like receptors, C-type lectins such as Dectin-1, and the glycosylphosphatidylinositol-anchored protein CD48.. Mast cells promote inflammation and other tissue changes in IgEassociated allergic disorders (Galli *et al., 2*008). IgE–allergen binding on FceRI on second encounter of allergen on mast cells triggers the release of preformed vasoactive mediators, synthesis of prostaglandins and leukotriene, and the transcription of cytokine these cause immediate-hypersensitivity reactions. In the bronchial mucosa, these induce mucosal oedema, mucus production, and smooth muscle constriction. In skin of atopic individuals it triggers both immediate-hypersensitivity and late-phase reactions (Bubnoff *et al., 2*002).

However, mast cells also can be important as initiators and effectors of innate immunity and adaptive immunity (figure 7)**.** At the earliest stages of infection, mast cells are important for communicating the presence of a pathogen by recruitment of multiple inflammatory cells including eosinophils (eotaxin), natural killer (NK) cells (IL-8), and neutrophils (IL-8 and TNF-a) (Gordon et al., 1990) and Mast cell–derived cytokines and chemokines can enhance the migration of dendritic cells (DCs; TNF-a and CCL20) and effector T cells (CXCL10/ IP10 and CCL5/RANTES) to the site of infection and to draining lymph nodes. Mast cell mediated enhancement of inflammation could induce damage of host tissues and worsen outcome during some infections, by producing pro-inflammatory IL-6, increase increased circulating histamine, causing increased mortality. Mast cells promote the activation of Th1/Th17 cells defence responses against *Leshmania. major* in mice. MCs triggered DC modulation promotes CD41 T-cell proliferation and polarization towards Th1 and Th17 responses (Dudeck *et al*., 2011). Mast cells can present antigen particularly for CD8+ T cells suggesting that mast cells function as antigen-presenting cells (Abraham *et al*., 2010**,** Galli *et al.,* 2006**).**

Mast cells play a pivotal role in the host defence against pathogens. Pathogen recognition by host receptors leads to mast cell activation and both direct and indirect antimicrobial responses (Urb *et al., 2*012, Abraham *et al., 2*010). They have both protective and detrimental role against various diseases (Woska *et al.,* 2012).

2.5.1 Mast cell signalling: Early events

The high affinity receptor FcεRI is expressed on mast cells as a heterotetrameric receptor composed of an IgE-binding α-subunit, the membrane-tetra spanning βsubunit and two identical disulphide-linked γ-subunits. The γ subunits are important for initiating signalling events 'downstream' of this receptor because each γ subunit contains one immunoreceptor tyrosine-based activation motif (ITAM). Antigen and IgE-induced crosslinking of cell surface FcεRI triggers activation of the kinase Lyn, which associates with β chain, phosphorylates which phosphorylates ITAM within β and γ chain receptor complex (Nadler *et al., 2*002). When phosphorylated, the β and γ chain ITAMs provide high-affinity docking sites for the SH2. The subsequent SYK- and/or LYN-mediated tyrosine phosphorylation of the transmembrane adaptor molecule LAT (linker for activation of T cells) is crucial for coordination of the downstream signalling pathways. The four terminal tyrosine residues in LAT (Y132, Y171,Y191 and Y226) are crucial and sufficient for the ability of LAT to regulate signalling in, and degranulation by, mast cells (Saitoh *et al*., 2003). The main signalling enzyme, which is regulated by both direct and indirect interactions with these tyrosine residues, is PLCγ. Fyn, that phosphorylates the adaptor Gab2 which activate PI(3)K pathway by binding its p85 subunit and recruiting it to its substrate lipids (Gu *et al., 2*001). Fyn and lyn initiates pathway synergize in late events at the level of PKC and calcium and regulate mast cell degranulation (Paricinni *et al., 2*002).

Phospholipase C-γ hydrolyzes phosphatidylinositol-4,5-bisphosphate to form soluble inositol-1,4,5-trisphosphate and membrane-bound diacylglycerol. The binding of inositol-1,4,5 triphosphate to its receptor in the endoplasmic reticulum rapidly induces the first stage of calcium (Ca2+) mobilization, which is the transient release of Ca2+ from endoplasmic reticulum stores; this in turn induces prolonged influx ofCa2+ through store-operated calcium release–activated calcium (CRAC) channels in the plasma membrane Ca2+ influx through store-operated channels is also dependent on the membrane potential, which is regulated by calcium-activated no selective cat ion channels such as TRPM4. TRPM4 activates a calcium activated no selective current that depolarizes membrane potential and limits the driving force for Ca2+ entry through CRAC channels in BMCMC. FcεRI-induced degranulation, release of leukotrienes and production of tumour necrosis factor (TNF), but not interleukin 6 (IL-6), Downstream of early FcεRI-induced signalling events (such as Ca2+influx), the final stages of mast cell degranulation require membrane fusion events (V Kalesnikoff *et al., 2*008, Malbec *et al* 1998, Suzuki *et al., 1*998, Olivera *et al* 2006).

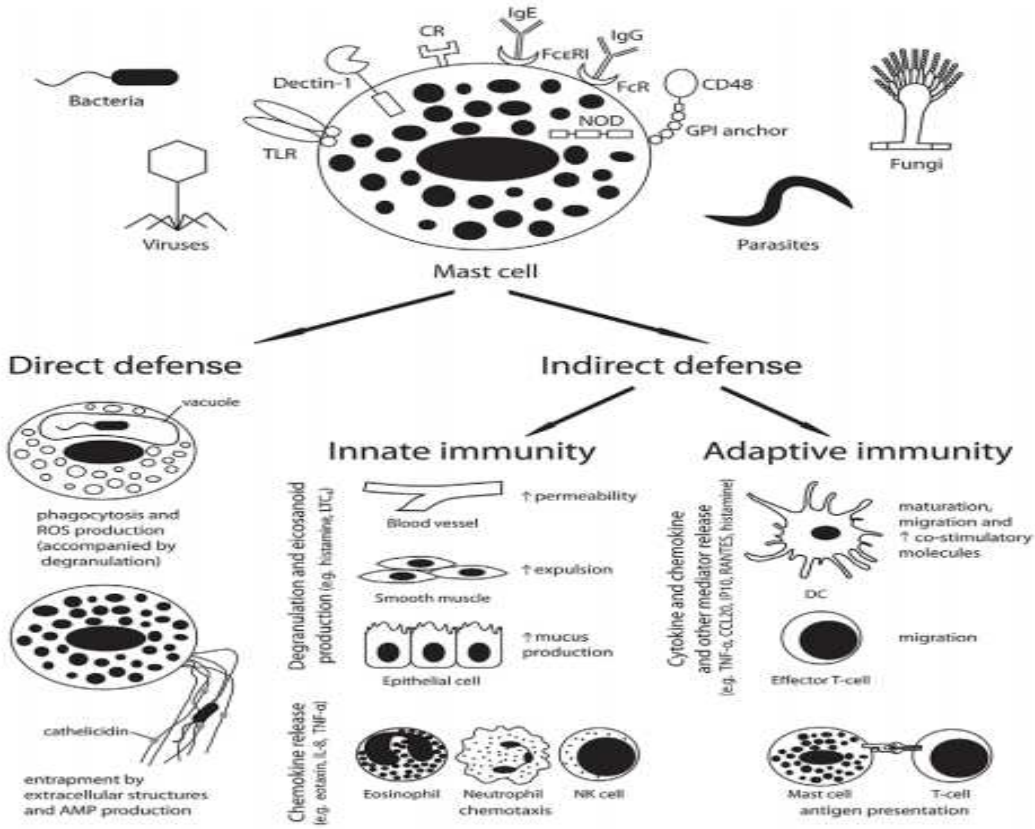


Figure 2.5: Mast cell's role in the host defence against pathogens

Pathogen recognition by host receptors leads to mast cell activation and both direct and indirect antimicrobial responses (Urb *et al., 2*012).

2.5.2 Mast cell degranulation: Later events

Mast cells are capable of releasing high amounts of their granular content in response to a single stimulatory event .The FcεRI-dependent cell activation process can result in both degranulation and *denovo* cytokine synthesis. Mast cells release of preformed mediators stored in the cell's cytoplasmic granules, e.g., vasoactive amines, neutral proteases, proteoglycans, and some cytokines and growth factors extracellularly, by a process called degranulation. Mast cells discharge their contents by compound exocytosis and in response to some stimuli by piecemeal degranulation (Galli *et al., 2*006, Abharam *et al., 2*010).The first study demonstrating SNARE-mediated contribution to mast cell degranulation was published in 1998 by the group of D. Castle (Guo *et al*., 1998).

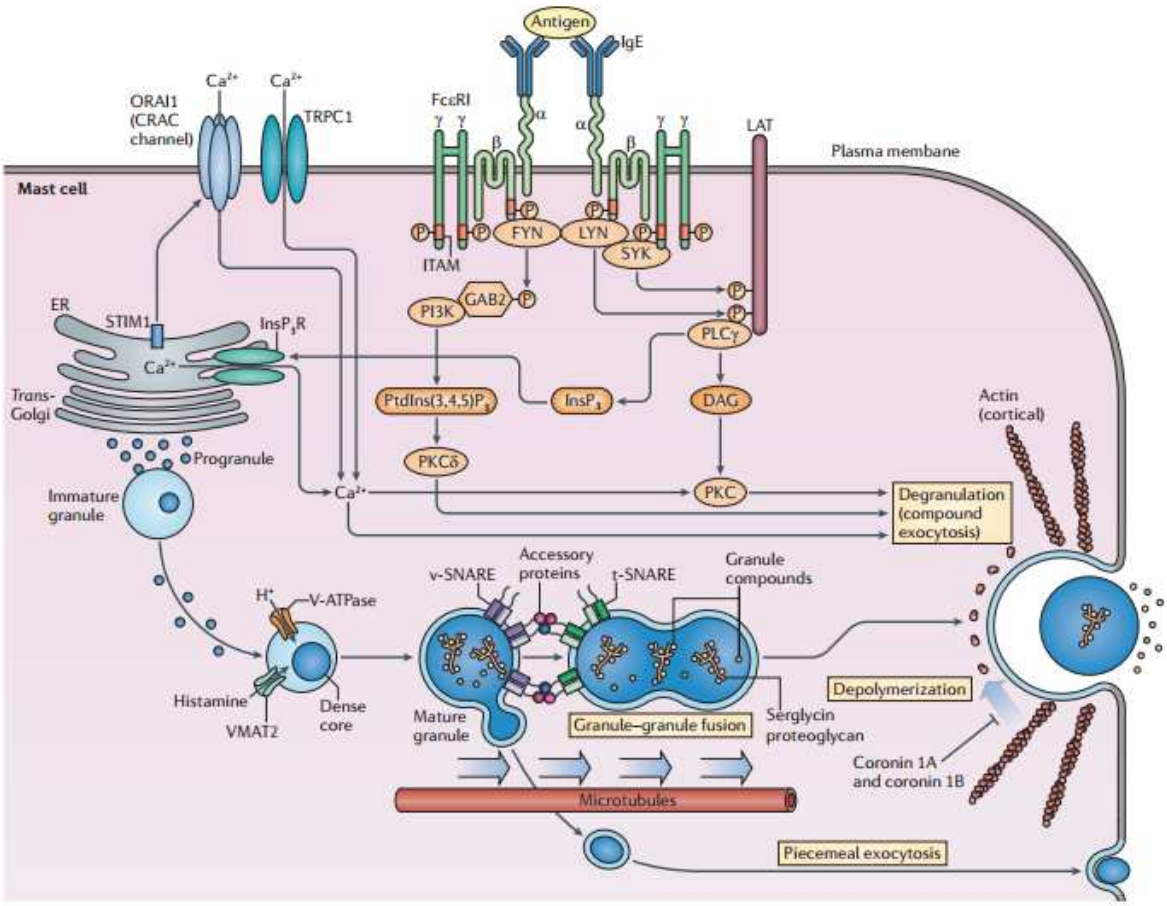


Figure 2.6: Simplified scheme for mast cell granule biogenesis, maturation and degranulation:

Mast cell secretory granule biogenesis is initiated at the *trans*-Golgi, where small vesicles bud off and then undergo multiple fusion events leading to the formation of immature granules.Granules then undergo maturation, a process in which dense core formation increases compounds such as proteases, bioactive amines and cytokines Mast cells express the high-affinity receptor for IgE (FcεRI) on their surface which have an antigen-binding α-subunit, a β-subunit that contains an immunoreceptor tyrosine-based activation motif (ITAM).When FcεRI-associated IgE molecules bind multivalent antigen (allergen) that leads to downstream signalling, that releases $Ca^{2+}$from the endoplasmic reticulum (ER). The increase in intracellular Ca2+ levels and the activation of PKC triggers the degranulation machinery so that granules translocate from the cell interior towards the plasma membrane in a microtubule-dependent manner. Degranulation is preceded by extensive granule–granule fusion events, which are mediated by various soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), including vesicle (v)-SNARE and target (t)-SNARE proteins, that are assisted by several accessory proteins. Likewise, the degranulation event depends on a distinct set of v-SNARE, t-SNARE and accessory proteins. Following degranulation, many of the preformed granule compounds (such as histamine) are released in a soluble form (Wernersson *et al., 2*014*).*

2.5.3 SNAREs in mast cells

Mast cells possess distinct secretory granule subsets, each with different cargo, different functions, and in some cases distinct SNARE proteins regulating their fusion with the plasma membrane. They express a wide array of SNAREs and their localization may differ between different cell types and species as shown in table 2.9. To date, described SNARE proteins in mast cells include the t-SNAREs SNAP-23 as well as STX2, 3, 4, and 6. VAMP family protein members include VAMP2, 3, 4, 7, and 8 (Sander *et al*., 2008). Primary murine mast cells have demonstrated that SNAP-23 and Syntaxin 4 localize to the plasma membrane, while Syntaxin 3, VAMP-2 and VAMP-8 appear to localize to secretory granules human mast cells have identified SNARE complexes composed of SNAP-23 and Syntaxin4 in complex with the R-SNAREs, VAMP-2. In RBL mast cells, SNARE complexes are present in lipid raft membrane micro domains. The association of SNARE complexes with these membranes is mediated primarily by their binding to SNAP-23. Ternary SNARE complexes selectively associate with lipid rafts during granule exocytosis from RBL mast cells (Puri and Roche *et al., 2*006).

Cell type	SNARE
RBL-2H3 cells	Qb,c family: SNAP-23
	Qa family: Syntaxin 2, 3, 4,
	R family: VAMP-1, 2, 3, 7, 8
	Qc family: Syntaxin 6
Primary murine mast cells	Qb,c family: SNAP-23, SNAP-25
	Qa family: Syntaxin 2, 3, 4,
	R family: VAMP- 2, 3, 4, 7, 8
Human mast cells	Qb,c family: SNAP-23
	Qa family: Syntaxin 1b, 2, 3, 4,
	R family: VAMP-2, 3, 7, 8
	Qc family: Syntaxin 6

Figure 2.7: Types of SNAREs present in different Mast cells

Source: Woska et al, 2012

2.5.3.1 The R SNARE

VAMP8 was the first SNARE protein examined for a role in cytokine/chemokine trafficking in mast cells. Expression of several VAMP (VAMP-1, -2, -3, -7, -8) family have been reported in multiple studies. In human mast cells (Sander *et al*).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 VAMP-8 is required for MC degranulation whereas, inhibition of VAMP-2 andVAMP-3 by TeNT or anti VAMP-2 and VAMP-3 antibodies did not alter histamine release (Sander *et al., 2*008). VAMP-8 regulates release of serotonin but not histamine from secretory granules of mast cells (Puri *et al*., 2008). *E. coli* leads to the inhibition of VAMP8 containing β-hexosaminidase granules (Frank *et al., 2*011). Endogenous VAMP-2 localizes to punctuate granular structures in RPMC and RBL cells. However, its direct co-localization with a granular marker has not been demonstrated (Guo *et al., 1*998, Paume *et al*., 2000). Another partner could be VAMP-8 that, likeVAMP-2, interacts with syntaxin 4 and co-localizes with a subset of secretory granules stained with an antibody to serotonin in RBL cells (Paumet *et al., 2*000) as VAMP-8 has been defined as an endosomal marker (Wong *et al., 1*998).

2.5.3.2 Qa SNARE

STX4, is localized at the plasma membrane of human mast cells, while in rodent mast cells it is found mainly on secretory granules. Q SNAREs STX-3 and SNAP-23 are required for chemokine release and that other Q SNAREs such as STX-6, STX- 4 and the R SNAREs VAMP-8 and VAMP-7 are partially involved (Frank *et al* 2011). Lorentz *et al*., 2014 has found that STX4 andVAMP8 play a specific role in CCL8 while anti-STX6 selectively inhibits CXCL8 and CCL2 and anti-VAMP7 CCL3.Similar to the findings observed in macrophages, the release of *de novo* synthesized cytokines by mast (Lorentz *et al., 2*014). Syntaxin 4 in RBL cells not only interacts with SNAP-23, but with another t-SNARE, but also with several v-SNAREs including VAMP-2, -3 and -8 (Galli *et al., 2*000).

2.5.3.3 Qb, Qc SNARE

Qb, Qc SNARE is expressed in all mast cells. In unstimulated mast cells, SNAP-23 is concentrated along with syntaxin 4 in foci corresponding to micro domains within the cell surface, and storage granules contain the partner SNAREs that serve in SNARE complex formation for granule-to-plasma membrane and granule-to-granule fusion (Castle *et al*, 2001). Upon stimulation, the relocation of SNAP-23 is a key event that facilitates t-SNARE complex formation and ensuing membrane fusion between the cell surface and peripheral granules and between granules throughout the storage pool (Guo *et al., 1*998). The carboxyl-terminal 23 amino acids of SNAP-23 is as important determinants for binding to VAMP but not to syntaxin (Vidhyanath *et al*, 2001). SNAP-23 is phosphorylated in the RBL mast cell line and in bone marrow-derived mast cells upon FcRI receptor cross-linking, the physiological trigger for mast cell degranulation. Ser95 and Ser120 are the two major phosphorylation sites of SNAP-23 in RBL mast cells (Hepp *et al*., 2005). These residues are located in the palmitoylated “linker” region separating the amino- and carboxyl-terminal coiled-coil domains of SNAP-23. IKK2-induces SNAP-23 phosphorylation at Ser120and Ser95, leading to IgE-induced degranulation in mast cells (Suzuki *et al*, 2008). The neuronal SNARE SNAP-25 shows weak mRNA expression in human mast cells, SNAP-23 is the consensus representative of the Qb, c family in both murine and human mast cells. SNAP23 hypo-phosphorylation induced by *E. coli* results decrease in SNARE complex (Wesolowski *et al., 2*014).

2.5.4 SNAREs interacting proteins of Mast cells

Mast cells were found to express the ubiquitous isoforms Munc18-2 and Munc18-3. In co-immunoprecipitation experiments Munc18-2 interacted with STX3 and less so with STX2, while Munc18-3 interacted with STX4 (Martin-Verdeaux *et al*., 2003). siRNA- mediated knock-down of Munc18-2 also blocked inflammatory mediator secretion in RBL mast cells (Tadokoro *et al*., 2007). Doc2 is expressed in both RBL-2H3 cells and BMMCs for lysosomal exocytosis. Doc2 interacts with Munc13-4 through the N-terminal region containing the Mid domain and the C-terminal region containing the C2B domain in a Ca2-independent manner Doc2 and Munc13-4 are colocalized on secretory lysosomes in RBL-2H3 cells and that Doc2 can interact with Munc13-4.

Complexin I and II are small soluble cytosolic proteins and interact with SNARE complexes RBL-2H3 cells express complexin II, but not complexin I (Tadokoro *et al*., 2005). Complexin II knock-down experiments revealed that complexin II positively regulated exocytotic release in mast cells by trans locating to the plasma membrane and enhancing the calcium sensitivity of the fusion machinery (Tadokoro *et al*., 2005). The association of complexin II with SNARE complex was not sufficient to trigger exocytotic membrane fusion. *In vitro* binding assays showed that complexin II interacts with SNARE complex containing STX3 to regulate mast cell degranulation, but does not bind to SNARE complex containing STX4.Several isoforms of synaptotagmin such as synaptotagmin II, III, V, or IX are expressed in mast cells (Baram *et al*., 2001, Haberman *et al*., 2007)

Synaptotagmin, one of the SNARE binding proteins, is a vesicular protein that has two C2 domains at its C-terminal (Pang *et al*., 2010). In neuronal cells, synaptotagmin 1 is thought to be a Ca2+ sensor for exocytotic release of neurotransmitters synaptotagmin 1 promoted liposomal membrane fusion mediated by neuronal SNARE through Ca2+ and PIP2 (Phosphatidylinositol 4,5-bisphosphate) (Wong *et al., 2*011). PIP2 is also involved in the enhancement effect of synaptotagmin 2 on SNARE-mediated membrane fusion. PIP2 localizes on the plasma membrane and may have the role of tethering secretory vesicles to the release sites to support the quick secretion after Ca influx (Takadaro *et al., 2*015).

It is now clear that mast cells uses a highly sophisticated machinery of proteins that enable the fusion of vesicles containing pre-stored and newly synthesized inflammatory mediators but still little of how these proteins are related to early signalling events, but still some answers remain to be answered such as basic connections like the phosphorylation of SNAP-23 by IKK and the responsiveness of certain processes to calcium signals are not known (Lorentz *et al* 2012). How SNAREs provide energy is applied to the membranes remains unclear (Rizo *et al., 2*012).

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plasmids and vectors used in this for cloning and expression system

The vector pEGFP-C2 (Clontech Laboratories, Inc. Genebank Accession # U57606) (figure 2), vector containing EGFP gene, including kanamycin and neomycin resistant gene were used in the experiments.pEGFP-C2 has been optimized for brighter fluorescence and higher expression in mammalian cells. The recombinant EGFP vector was transfected into mammalian cells using any standard transfection method and can also be used simply to express EGFP in cell line of interest as a transfection marker. The plasmid pEGFP-C2 syntaxin 4 was previously cloned was used in the study and the sequence was analyzed.

Also, the expression vector, pDsRed1-C1 (Clontech Laboratories, Inc) in figure 3, a novel red fluorescence protein that is optimized for high expression in mammalian cells was used in the study.pDsRed1-C1.pDsRed1-C1 can be used to construct fusions to the C-terminus of pDsRed 1 and the fusion to construct retaining the fluorescent properties of native DsRed1 protein and its expression and localization is monitored by flow cytometry and fluorescence microscopy. The plasmid pDsRed1-C1 syntaxin 4 which was previously cloned was used in the study and the sequence was analyzed.

3.1.2 Enzymes, chemicals and reagents

Molecular biology reagents-chloroform, isopropanol, ethanol, 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 obtained from NEB (Quick Ligase).DNA ladders were from NEB and Fermentas (Figure 3).The restriction enzymes used in this study were obtained from New England Bio labs (NEB) Inc. UK, enhanced chemiluminescence (ECL) from Millipore.

RPMI 1640 Media, Minimal Essential Medium (MEM) and Iscove's media, Trypsin, Phosphate Buffered Saline (PBS), Sheath Fluid, Fetal Bovine Serum (Gibco), Trypan Blue were obtained from SIGMA.

Table 3.1.: List of enzymes and their restriction sites

Enzyme	Recognition site	Supplied NEB Buffer
EcoRI-HF	GAATTC	Cut smart
Apa I	GGGCCC	Cut smart
Bam HI	GGATCC	Cut smart

Tools used for designing and validating primers and analyzing sequences

Table 3.2 Tools list for designing, validating primers and analyzing sequences after sequencing.

Software	**Links**
Oligo calc (Oligonucleotide properties calculator)	http://biotools.nubic.northwestern.edu/OligoCalc.html
OligoEvaluator	http://www.oligoevaluator.com/OligoCalcServlet
Multalin(MSA by Florence Carpet)	multalin.toulouse.inra.fr/multalin/
Sequence editor	http://www.fr33.net/seqedit.php
Extended Nuclic Acid Sequence Massager	http://www.cmbn.no/tonjum/seqMassager-saf.htm
Expasy	http://web.expasy.org/translate/

3.1.3 Primers

The primers were designed for SNAP 25 (635 bp) and GAPDH (180bp) using guide lines for rat primer designing and then were tested for various parameters like length of primer, GC content, Tm, self-complementation, hair pin formation etc using different tools given below. The primers were designed in such a way that they contain restriction enzyme site and do not shift the reading frame of fusion protein. Primer for rat GAPDH gene was designed using primer-BLAST-NCBI.GAPDH PCR was done as positive control.

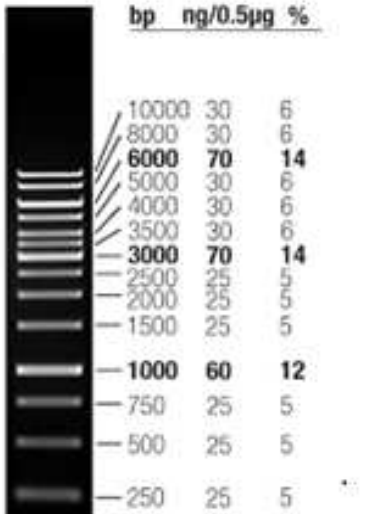
Table 3.3 **List of primer sequences used for PCR amplification**

NAME	SEQUENCE	LENGTH	GC%	Tm(ºC)
SNAP-25B FP(*EcoRI* site)	5’ AAGAATTCATGGCCGAGGACGCAGAC 3’	26	57.7	62
SNAP-25B RP(*BamHI* site)	5’TCCGGATCCTTAACCACTTCCCAGCATCTTTGT3’	33	45.5	64.9
GAPDH(FP)	5'GCGAGATCCCGCTAACATCA3'	20	55	59.97
GAPDH(RP)	5'CTCGTGGTTCACACCCATCA3'	20	55	59.97

Where,XXX is restriction site,XXX is linker region and XXX is coding region.

Markers used for restriction analysis

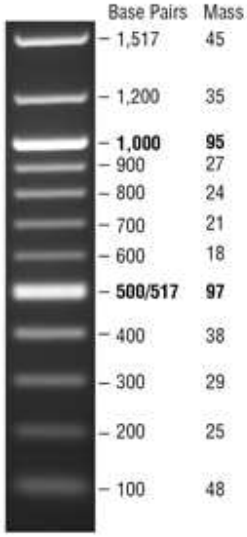
(a)



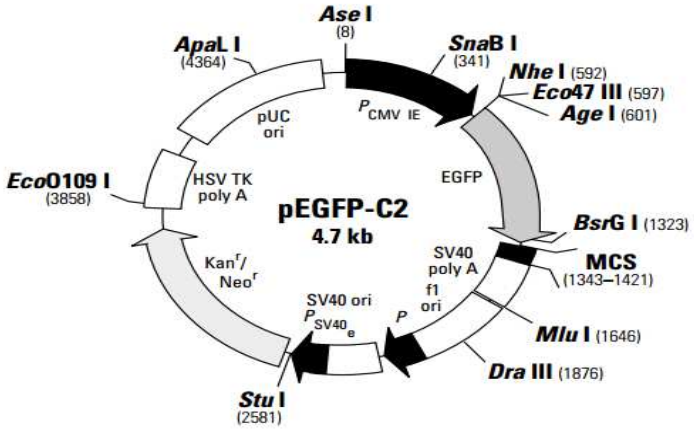
pEGFP-C2 Vector Information

GenBank Accession #: U57606

(b)



100 bp DNA Ladder visualized by ethidium bromide staining on a 1.3% TAE agarose gel. Mass values are for 0.5 µg/lane.



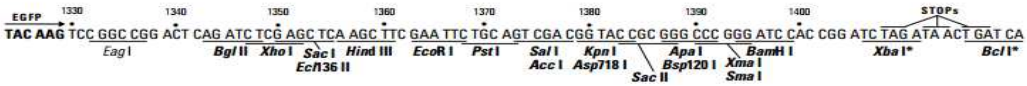
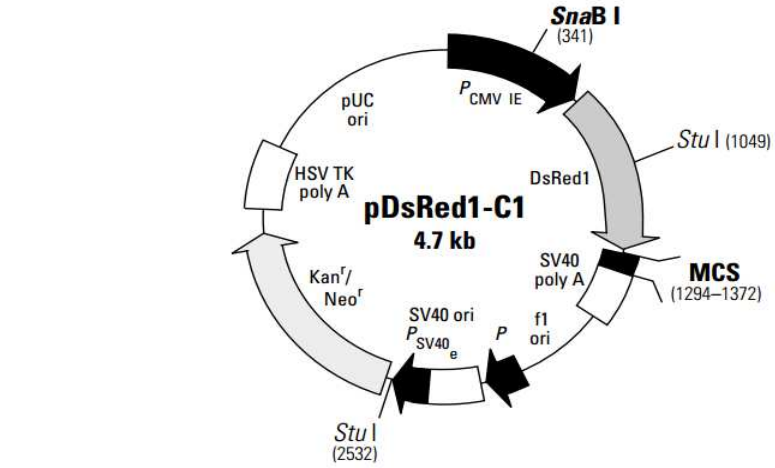
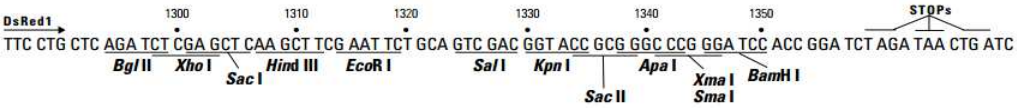


Figure 2: Restriction map and MCS of pEGFP-C2 vector

pDsRed1-C1 Vector Information PT3425-5

Catalog #6924-1





3.1.4 Cell cultures

The rat basophilic leukemia (RBL-2H3) mast cell was a kind gift from 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 as sub confluent monolayers at 37°C in humidified atmosphere containing CO_2 and passaged with trypsin.

PC 12 (pheochromocytoma (ATCC® CRL-1721)). They are maintained in RPMI 1640 media containing 5% Fetal Bovine Serum (Gibco) and 10% Horse Serum (Gibco) and 1% penstrep antibiotic (Invitrogen) and were maintained at 37°C in humidified atmosphere containing 5% CO_2.

3.2 Methods

3.2.1 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 were streaked on LB plates and incubated overnight at 37°C. A single colony was picked and inoculated in 3ml of LB broth and incubated overnight at 37°C in a shaker incubator. Inoculum of 200 µl of overnight grown cells was taken to obtain culture at log phase by inoculating in 50ml LB media in a flask. The flask was incubated at 37°C till optical density at 600nm reached 0.5-0.6, taking LB broth as control. Cells were kept on ice for 5-10 mins. Then cells were collected by centrifugation at 6000 rpm at 4°C for 10 mins. Supernatant was drained off completely and obtained cell pellet was resuspended in 24 ml of freshly prepared 0.1M $MgCl_2$ and 6 ml of 0.1M $CaCl_2$. The pellet was

dissolved properly by gentle pipetting and incubated on ice for 5 min. The suspension was centrifuged at 6000 rpm at 4° C for 10 mins and obtained pellet was resuspended in 1.5 ml ice cold 0.1M $CaCl_2$. Tubes were sealed with paraffin and kept overnight at 4°C. The competent cells were stored at 10% glycerol stocks in small aliquots in eppendorf tubes and stored at -80° C. The competence efficiency of prepared competent cells was then checked by transformation.

3.2.2 Transformation of competent E.coli with plasmid construct

Chemically competent cells (*E.coli* DH5α) usually containing 75µl per tube were stored at -80° C until use. The stored competent cells were thawed keeping on ice for 10 mins. Briefly, 10-100ng of DNA was added to 75µl competent cells in 1.5ml eppendorf tube, gently mixed by pipetting and further incubated at 15 mins on ice. Cells were briefly heat shocked at 42° C for 60secs in water bath and then immediately chilled on ice for 2 mins. Tubes were removed from ice and to mixture, 925µl LB broth medium was added and cells were allowed to grow at 37° C for one hour in a shaker incubator at 225rpm. The *E.coli* cultures were centrifuged at 10,000rpm for 5 mins and 925µl of the supernatant was removed. The pellet was resuspended in 75µl LB broth medium and plated on kanamycin (50µl/ml of media) containing LB agar plate and incubated overnight at 37° C. Small colonies that were able to grow on media selected with kanamycin after incubation would have been transformed by the plasmid (Sambrook and Russel., 2001)

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

$$\textbf{Transformation efficiency} = \frac{\text{Total number of colonies formed}}{\text{Amount of plasmid DNA added in µg}} \times 1000$$

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

3.2.3 Mini preparation of plasmid DNA

Mini preparation of plasmid was carried out by alkaline lysis method (Sambrook and Russel, 2001). A single colony was picked of *E.coli* containing desired plasmid and inoculated in 5ml LB media in a 15 ml tube containing kanamycin (50µg/ml) and grown over night at 37°c at 225 rpm. Cells were harvested by centrifuging 1.5 ml of culture at 12,000rpm for 30secs. The media was aspirated and another 1.5ml of culture was added and centrifuges at 12,000rpm for 30secs.

The bacterial pellet was resuspend in 100µl ice cold solution I by vigorous vortexing and 0.5µl RNase (stock 10mg/ml) was added and incubated on ice for 2 mins. The tubes were inverted after adding200 µl of freshly prepared solution II tube 5-10 times. It was incubate on ice for 3mins. The tubes were inverted after adding 150 µl ice cold solution III and incubated on ice for 5min. It was centrifuged at 12,000rpm for 10 mins at 4°C (to remove cell debris). Supernatant was taken and 0.6 volume of isopropanol was added and incubated on ice for 5 mins. It was centrifuged at 12,000rpm for 5 mins. Supernatant was drained and DNA pellet was collected and rinsed by adding 50µl of 70% ethanol. It was centrifuged at 12,000 for 5 mins and ethanol was removed by carefully pipetting. DNA was air dried at 37°C till last drop of alcohol dries out. The pellet was then dissolved in 50µl of 10 mM Tris-Cl (pH-8) and kept in water bath for 10 mins at 37° C for 10 mins. Plasmid was then stored at -20° C.

3.2.4 Maxi prep of plasmid by alkaline lysis

3.2.4.1 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 containing appropriate antibiotic (kanamycin 50µg/ml of media) in a 15ml falcon and grown overnight at 37^{o} C with shaking at 225 rpm. From log phase 2.5ml of primary culture was transferred into 500ml LB media containing antibiotic in a 2 liter conical flask and grown for 16-18 hours at 37^{o}C with shaking at 250rpm. The cells were then harvested by centrifugation at 6000rpm for 15 mins at 4^{o}C in Hiatchi R_{10} A3 rotor. The supernatant was drained off and pellet was resuspended in 50ml of ice cold STE. The solution was centrifuged at 6000rpm for 15 mins at 4^{o}C.

3.2.4.2 Alkaline lysis

The pellet was resuspended in 18ml ice cold solution I with vigorous shaking and 20µl of RNase (10mg/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 mins. To the cell suspension 15ml of ice cold solution III was added and mixed thoroughly by gentle inversion of the tubes for many times and then incubated in ice for 10mins. The whole content was centrifuged at 9000 rpm for 4^{o}C to recover bacterial lysate and supernatant was filtered through cheese cloth. To the filtrate obtained, 0.6 volume of isopropanol was added and mixed by inversion and then incubated at room temperature for 10 mins. Tubes were then centrifuged at 9000 rpm for 20mins at room temperature. The pellet was washed with 70% ethanol and then centrifuged at 9000 rpm for 5 mins. The DNA pellet was allowed to air dry and then resuspended in 1.5ml of 10mM Tris (pH8) at 37^{o}C and finally stored at -20^{o}C (Sambrook and Russel., 2001).

3.2.5 Determination of concentration and purity of DNA using Nano drop ND2000 Spectrophotometer

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

DNA purity ratio= A260/A280

Where, A260 and A280 are optical density (OD) of DNA sample determined at wavelengths 260nm and 280nm 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 260nm provides an estimate of the concentration of the nucleic acid in the sample.

Concentration of plasmid DNA=50x A260nm x dilution factor

Where, A260=optical density of plasmid DNA determines at 260nm. An OD 260 of 1 corresponds to approximately 50µg/ml of DNA.

3.2.6 RNA Isolation

All equipment, plastic ware used for RNA isolation were treated with DEPC water to remove RNA contamination. RNA isolation of PC12 cell culture in RPMI (5% FBS +10%HS) in 1% pen strep antibiotic was carried out in its log phase of growth using TRI reagent (SIGMA as per manufacture's instruction. Form the cultured cells, 5 million cells were harvested by centrifugation process at 1200 rpm for 5 mins and washed twice with PBS. The cells were Suspended in 1ml of Tri reagent and vortexed vigorously. Chloroform was added at 0.2ml per ml of Tri reagent. The solution was allowed to stand at room temp for 15 mins and then centrifuged at 12000g for 15mins at 4^0C.

Three distinct layers were formed in the eppendorf tube. The upper aqueous phase was carefully taken and transfered into a fresh tube and 0.5ml of isopropanol per ml of Tri reagent was added and mixed to precipitate RNA. The solution was allowed to stand at room temperature for 15 mins and it was centrifuged at 12000g for 10mins at 4^oC. The supernatant was discarded carefully and RNA pellet was washed with 75% ethanol by centrifugation at 7500g for 5 mins at 4^oC. Ethanol was removed by air drying for 30 mins at RT. It was resuspend in TBE buffer and incubate in TBE buffer for 10 mins at 70^0C. 1µl of (40u/µl) RibolockRNase inhibitor was added and stored at -20^oC.

3.2.7 Determination of Concentration and purity of RNA Spectrophotometer

The purity and concentration of the isolated RNA sample was checked by measurng absorbance at 260nm and 280 nm.Pure preparation of RNA has)OD_{260} /OD_{280} values of ≥1.7

3.2.8 Formaldehyde agarose gel electrophoresis for RNA

Formaldehyde agarose gel was prepared at the concentration of 1.2% by boiling 1.2 gm agarose powder in 100 ml 1X formaldehyde agarose gel running buffer and 1.5µl (0.15µg/ml) ethidium bromide was added after cooling to 60^o -70^o C. Thereafter the gel was cast and was put into gel tank containing 1X formaldehyde agarose gel running buffer. RNA samples (1 vol 6X RNA loading dye +2 volume of RNA and 3 volume of autoclaves MilliQ) were loaded, voltage 90V was supplied for 2 hours and visualized under Biorad GELDOC system.

3.2.9 Reverse Trasncriptase PCR

cDNA was synthesized from 3µg of RNA using M-MuLV Reverse transctiptase enzyme(NEB) in reaction mixture of 20µl. Reaction mixture was prepared in 0.2ml PCR tube by mixing nuclease free MQ H_2O(9.5µl), M-MLV RT 5X buffer (4µl), RNA template (2.5µl), oligo(dT) (1µl), 2µl dNTPs (Thermoscientific) and 1 µl M-MuLV Reverse Transcriptase was added to the mixture and reverse trasncription was carried out for 1h at 37^o C. the CDNA gel was run onto 1.5% agarose gel containing ethidium bromide (0.15 µg/ml) for 2 hours and then visualized under Biorad GELDOC system.

Table 3.4: PCR mixture and reaction conditions of GAPDH

Components	Stock Conc	Final Conc	Volume
MQ H_2O	-	-	17.8 µl
Std. Taq Reaction buffer(NEB)	10X	1X	2.5µl
dNTPs	10mM	200 µM	0.5 µl
Forward Primer	10 µM	0.4 µM	1 µl
Reverse Primer	10 µM	0.4 µM	1 µl
cDNA	-	-	2 µl
Taq pol(NEB)	5 U/µl	1 U	0.2 µl
Total volume	-	-	25 µl

Condition	Temperature	Time	Cycles
Initial Denaturation	95°C	3 mins	-
Denaturation	95°C	30 secs	35 cycles
Annealing	58°C	30 secs	
Extension	72°C	1 min	
Final elongation	72°C	15 mins	-
Hold	4 °C	-	-

Table 3.5: PCR mixture and reaction mixture for SNAP 25

Components	Stock Conc	Final Conc	Volume
MQ H_2O	-	-	16.3 µl
Std. pfu Reaction buffer(NEB)	5X	1X	5 µl
dNTPs	10mM	2.4mM	0.5 µl
Forward Primer	10 µM	0.2 µM	0.5 µl
Reverse Primer	10 µM	0.2 µM	0.5 µl
cDNA	-	-	2 µl
pfu Enzyme(NEB)	5U/µl	1 U	0.2 µl
Total volume	-	-	25 µl

Condition	Temperature	Time	Cycle
Initial Denaturation	98°C	3 mins	-
Denaturation	98°C	30 secs	
Annealing	58°C	1 min	35 cycles
Extension	72°C	45 secs	
Final elongation	72°C	15 mins	-
Hold	4 °C	-	-

3.2.10 PCR purification

QIAGEN, PCR Purification Kit was used for purification of PCR products. Buffer PB (5 volumes) was added to 1 volume of the PCR reaction mix and mixed. A QIAquick spin column was placed in a 2 ml collection tube and the sample was added into the QIAquick spin column, and centrifuged for 13,000rpm at 1 min. Flow-through was discarded and QIAquick spin column was placed back in the same collection tube. For washing purpose 0.75 ml PE buffer was added to the QIAquick spin column and centrifuged for 13,000 rpm at 1 min. Flow through was discarded and QIAquick spin column was centrifuged for 13,000rpm at an additional 1 min to remove residual wash buffer. QIAquick column was placed into a clean 1.5 ml micro centrifuge tube. To elute DNA, 30 µl of Buffer EB (10mM TrisCl, pH 8.5) or warm water (pH 7-8.5) was added to the centre of the column and centrifuged again for 13,000rpm at for 1 min. All centrifugation steps were carried out at ≥ 17,900 × g (~ 13,000 rpm) in a conventional table-top microcentrifuge (Eppendorf).

3.2.11 Restriction digestion for conformation of syntaxin 4 containing plasmid

In all cloning experiment, the vectors pEGFP-C2 STX4 and pDsRed1-C1 STX4 were cut by double digestion by the appropriate restriction enzymes *EcoRI HF* and *ApaI* with cut smart buffer. The DNA fragments to be analyzed were produced by restriction digestion of the of vectors separately. The restriction digestion mixture was set up as

Table 3.6 Restriction digestion mixture for plasmids containing syntaxin 4

Autoclaved milli Q	16.6µl	Autoclaved milliQ	16.6 µl
CutSmart buffer (NEB)	2 µl	Cutsmart buffer (NEB)	2 µl
ApaI	0.2 µl	*ApaI*	0.2 µl
EcoRI-HF	0.2 µl	*EcoRI-HF*	0.2 µl
pEGFP-C2 STX4	1 µl	pDsRed1-C1 STX4	1 µl
Total	20 µl	Total	20 µl

The digested samples were mixed and tubes were spun briefly and then incubated at 37°C for 2-3 hours and then the samples were run in gel.

3.2.12 Restriction digestion of pEGFP plasmid and SNAP 25 gene for cloning

Table 3.7: Restriction digestion mixture for double digestion of SNAP 25 and pEGFP

Autoclaved milli Q	16.6µl	Autoclaved milliQ	16.6 µl
CutSmart buffer (NEB)	2 µl	Cutsmart buffer (NEB)	2 µl
BamHI	0.2 µl	*Bam HI*	0.2 µl
EcoRI-HF	0.2 µl	*EcoRI-HF*	0.2 µl
pEGFP-C2	1 µl	SNAP 25	1 µl
Total	20 µl	Total	20 µl

The digested samples were mixed and tubes were spun briefly and then incubated at 37^{o}C for 2-3 hours and then the samples were run in gel.

3.2.13 Agarose gel electrophoresis for digested plasmid DNA

Agarose gels were made containing 0.8% agarose in 0.5X TBE (0.8 gram of agarose powder in 100 ml of TBE buffer) placed in a 250 ml conical flask 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 (0.15µg/ µl) of ethidium bromide was added to it. Following quick mix gel cassette was prepared and allowed to solidify after which gel was transferred to DNA gel tank containing 0.5X TBE.

DNA samples were loaded as:

4 µl 6X loading dye + 20 µl restriction digestions sample $\longrightarrow$ loaded 10 µl in each well

1 µl of undigested DNA + 5 µl Autoclaved MQ+ 1 µl 6X loading dye $\longrightarrow$ loaded 6 µl in a well

1 µl 1kb Gene ruler maker+ 5 µl Autoclaved MQ+1 µl 6X loading dye $\longrightarrow$loaded 6 µl in a well

The gel electrophoresis was performed at 90 volts for 3 hours and the gel was observed under Biorad GELDOC system.

3.2.14 Gel electrophoresis and DNA gel extraction from gel

Firstly the samples were run along with marker on 0.8% agarose gel. Inorder to extract the DNA band of interest, agarose gels were firstly examined under UV light in a spectrophotometer and a clean scapel was used to excise the correct DNA band. The excised gel band was then transferred into a sterile, pre-weighted 2ml 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. The gel slice melted at 50^{o}C for 10mins 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 QIAquick spin column (800µl of solution at a time) and

centrifuged at 13000 rpm for 1 min at RT. The flow through was discarded and QIAquick column was kept back into the same collection tubes. Buffer QG (5volume) was added to the column for complete removal of all the traces of agarose and centrifuged at 13000 rpm for 1 min. Then 750µl of buffer PE containing ethanol was added to column in order to wash it and centrifuged at 13000 rpm for 1 minute. The flow though was discarded and to make sure all ethanol was removed, the column was centrifuged for extra 1 min. Finally the column was placed into 1.5 ml eppendorf tube and 30µl of warm buffer EB was added to the centre of the column and allowed to incubate for 5 mins at RT. The column was centrifuged at 13000 rpm for 1 min to collect the DNA which was stored at -20^{o}C.

3.2.15 DNA ligation

The vector and insert were then mixed at a ratio of 1:5 respectively using formula given below and ligated using a standard ligation.

$$\textbf{ng of insert} = \text{ng of vector to be added} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio}$$

The complementary overhangs of the vector and the insert were ligated under the action of T4 DNA ligase enzyme (NEB).The ligation mixture was set up as

Table 3.8: Restriction digestion mixture for double digestion of SNAP 25 and pEGFP

	Initial conc.	Final conc.	
Insert SNAP 25	125ng/ µl	50ng	8 µl
Vector	136ng/ µl	34ng	5 µl
Nuclease Free Water			4 µl
10x T4 ligation Buffer	10X	1X	2 µl
T4 DNA ligase			1 µl
Total			20µl

Samples were mixed and tubes were spun briefly and then incubated at 16^{o}C overnight. Immediately, after ligation half of the ligation mixture was used to transform *E.coli* DH5α competent cells as the method described previously.

3.2.16 Colony PCR

Single isolated transformed colonies were picked up with the help of toothpick and transferred to the reaction mix prepared as follows and PCR was run in the same conditions as before.

Table 3.9: PCR mixture and reaction conditions of SNAP 25 by colony PCR

Components	Stock Conc	Final Conc	Volume
MQ H_2O			19.8 µl
Std. Taq Reaction buffer(NEB)	10X	1X	2.5µl
dNTPs	10mM	200 µM	0.5 µl
Forward Primer	10 µM	0.4 µM	1 µl
Reverse Primer	10 µM	0.4 µM	1 µl
Bacterial colony			-
Taq pol(NEB)	5 U/µl	1 U	0.2 µl
Total volume			25 µl

Condition	Temperature	Time	Cycles
Initial Denaturation	95°C	5 mins	
Denaturation	95°C	30 secs	
Annealing	58°C	30 secs	35 cycles
Extension	72°C	1 min	
Final elongation	72°C	15 mins	
Hold	4 °C	-	

3.2.17 Cell culture of RBL cell line

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 sub cultured by trypsinisation.

PC 12 pheochromocytoma (ATCC® CRL-1721), They are maintained in RPMI 1640 media containing 5% Fetal Bovine Serum (Gibco) and 10% Horse Serum (Gibco) and 1%penstrep antibiotic (Invitrogen) and were maintained at 37°C in humidified atmosphere containing 5% CO_2 and cells were harvested by centrifugation and counted on haemocytometer using Trypan blue staining .

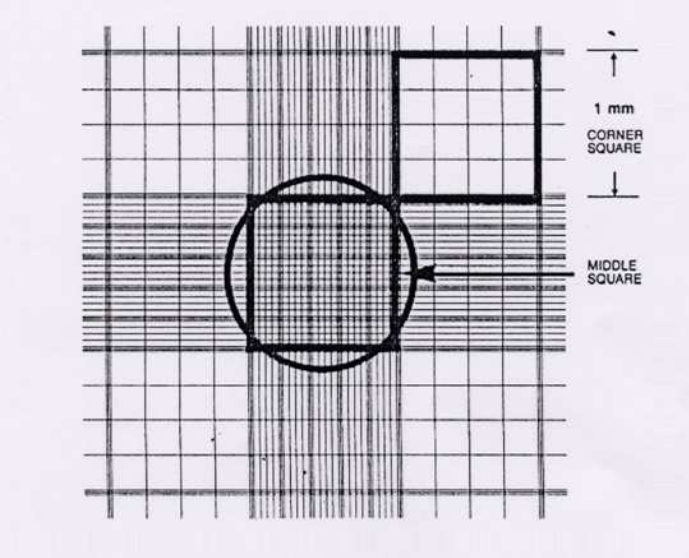


Figure 3.1: Haemocytometer

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. Each one mm square is divided into 25 medium sized squares (groups)(0.2mm x 0.2mm each), each of which is further subdivided into 16small squares (0.05 x 0.05mm each), thus, a total of 400 squares in 1mm. Each large square has a volume of $1x1x0.1mm=10^{-4}cm^3$ (Strober *et al.,* 2001).

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 Diluted factor x 10^4

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

3.2.18 Transfections of RBL-2H3 cells

3.2.18.1 DNA preparations

In a sterile eppendorf, 20µg DNA , 180 µl nuclease free water, 0.5 µl glycogen,20 µl alkaline solution III and 500 µl chilled 70% ethanol were mixed by inversion and incubated overnight at -20^{o}C (Puri *et al*., 2003). The whole of the mixture was then centrifuged at 13000 rpm for 30 mins at 4^{o}C. The pellet was washed with 50 µl of 70% alcohol and centrifuged at 13000 rpm for 8 mins 4^o C.

The pellet was dried properly and then dissolved in 25 µl of nuclease free water. The mixture was kept at 37^{o}C and immediately used for transfection.

3.2.18.2 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 antibiotic free RBL medium. Cells were counted in haemocytometer and re suspended in serum free and antibiotic free RBL media. Five million cells in 0.5ml medium were transfected by electroporation 320V, 950µF, Bio-Rad Gene Pulsar) using 5-20µg DNA. Transfected cells were immediately plated in pre warmed antibiotic-free RBL medium in tissue-culture dishes and also in cover slips containing dishes. It was then analysed after 24-48hrs. 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 and fluorescence intensity in red (FL2)

3.2.18.3 Flow cytometer

Cells were harvested after 24-48hrs by trypsinization. Cells were washed twice with antibiotic-free RBL medium and once with PBS containing 1% FCS. Cells were re-suspended in PBS analysed by BD FACS Calibur.Ten thousand 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 channel and fluorescence intensity in red (FL2).

3.2.18.4 Immunoflourescence microscopy

RBL cells plated on coverslips after transfection were fixed with 4% PFA for 20-30mins at RT after 6-24hrs. They were then washed twice with 50mM NH_4Cl in PBS to remove excess of paraformaldehyde. It was again washed twice with PBS. Coverslips were mounted in Fluor mount G and images were collected in Nikon Eclipse Ti microscope at 60X magnification.

3.2.19 DiIC 16 staining on adherent RBL-2H3

RBL cells were plated on coverslips after transfection with desired plasmid. Cells were washed twice with ice cold PBS. DiIC 16 (50µg/ml) was added in ice cold. It was incubated on ice for 10 mins and then washed three times with ice cold DMEM complete medium(10% FCS). It was again washed twice with ice cold PBS. Cells were fixed with 4% PFA for 20-30mins at RT. They were then washed twice with quencher. It was again washed twice with PBS. Coverslips were mounted in Fluoromount G and images were collected in Nikon Eclipse Ti microscope at 60X magnification.

3.2.20 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western Blotting

Exponentially growing RBL cells (10 million/ml) were taken and lysed in 0.2% triton X and lysis buffer. Cells were incubated in ice for 1 hour and then debris separated by centrifugation at 13000rpm for 30 secs. The lysates were prepared in SDS-PAGE sample buffer and heated at 95^{o}C for 5 min. The samples were then loaded onto precast 10.5% and 4.5% SDS-PAGE gels and electrophoresed at 80 V until gel run up to stacking and at 120 V in resolving gel till the dye front reaches the end. Proteins were transferred from the SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes at 320 mA for 90 mins using the transfer buffer system at RT. The membranes were blocked overnight in PBS/T/M (20 ml) in cold room in a smooth scratch free plastic box followed by two times washing with 20 ml PBS/T at RT for 15 mins each. The

membrane was then incubated for 60 mins with proper dilution of primary antibody in PBS/T at RT. The membranes were washed two times for 15 min each with PBS/T/M and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at room temperature. Again, the membranes were washed two times with PBS/T/M and two times with PBS/T for 15mins each before immunoreactive proteins were visualized by incubating the membranes with enhanced chemiluminescence (ECL) reagents for 1 min and then exposing the membranes to X-ray film for different time periods. Prior to the reprobing of membranes with additional antibodies, the membranes were washed two times with 20 ml PBS/T for 15mins each and then treated with 20 ml PBS/T/M in cold room for 30 mins. The same way was processed for reprobing with another antibody.

CHAPTER 4 RESULTS

4.1 Expression of syntaxin 4 in RBL cell line, rat brain homogenate and spleen homogenate

Presence of syntaxin 4 was examined by western blotting technique in RBL cell line. Homogenate of brain and spleen was taken along with RBL cell lysate. Immunolotting of all of these were performed using polyclonal rabbit antibody of syntaxin 4 (synaptic system) and goat anti rabbit HRP as secondary antibody. Immunoblotting of anti syntaxin 4 clearly reveals the presence of syntaxin 4 on RBL lysate, rat brain and spleen. These blots show that syntaxin 4 is present RBL cells.

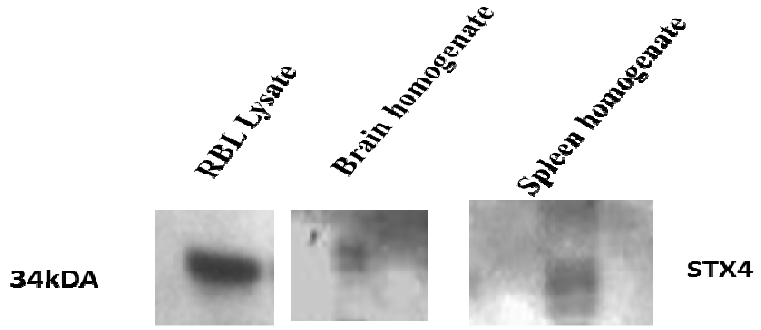


Figure 4.4.1: Immunoblot of syntaxin 4 in RBL, brain and spleen of rat Syntaxin 4.

Expression on RBL cells lysate, rat brain and rat spleen by resolving in SDS-PAGE and immunoblotted with anti syntaxin 4 antibody.

4.2 Devising a strategy to study the fall out of syntaxin 4 from pEGFP syntaxin 4 and pDsRed1 syntaxin 4 plasmids.

The plasmids used for this study was pEGFP syntaxin 4 and pDsRed syntaxin 4 were previously cloned in both the vectors using the restriction sites of *EcoRI* and *ApaI*. pEGFP syntaxin 4 and pDsRed were confirmed by restriction digestion was performed using *Ecori*-HF and *ApaI*. Then, the plasmids were sent for sequencing and sequences were analyzed using multalign software.

4.2.1 Maxi prep of pEGFP STX 4 and pDsRed STX 4

The amount and purity the plasmid was increased by maxi prep of both pEGFP STX 4 and pDsRed STX 4. After plasmid extraction 3778.3 ng/µl of pEGFP STX4 and 3010.7ng/µl of pDsRed STX4 was obtained. The transformation efficiency in case of pEGFP STX 4 was found to be 4.5 x 10^6 and in case of pDsRed STX 4 it was found to be 3.9 x 10^6 (Table 1). The extracted plasmids concentration and purity was checked by Nano drop (ND 2000) reading (Table 2).

4.2.2 Restriction digestion of pEGFP STX 4 and pDsRed STX 4

Syntaxin 4 was previously ligated in *EcoRI* and *Apa I* site into pEGFP and pDsRed so the plasmids fused with syntaxin 4 were confirmed by double digestion. Both the size of pEGFP syntaxin 4

and pDsRed syntaxin was found to be 5.5 kb when single digested with *ApaI*. The size of syntaxin 4 was found to be 897 bp by double digestion with *EcoRI* and *Apa I*.

Table 4.1: Determination of transformation efficiency

S.No	Plasmid used	Amount of plasmid DNA(µg)	No. of colonies observed	Transformation efficiency
1	pEGFP STX 4	100×10^{-3}	450	4.5×10^{6}
2	pDsRed STX 4	100×10^{-3}	390	3.9×10^{6}

Table 4.2: Quantification and determination of purity of DNA by nanodrop ND 2000

S.No	Plasmid DNA	Concentration (ng/µl)	Purity ratio (A260/280 ≥1.8)
1	pEGFP STX 4	3778.3	1.96
2	pDsRed STX 4	3010.7	1.83

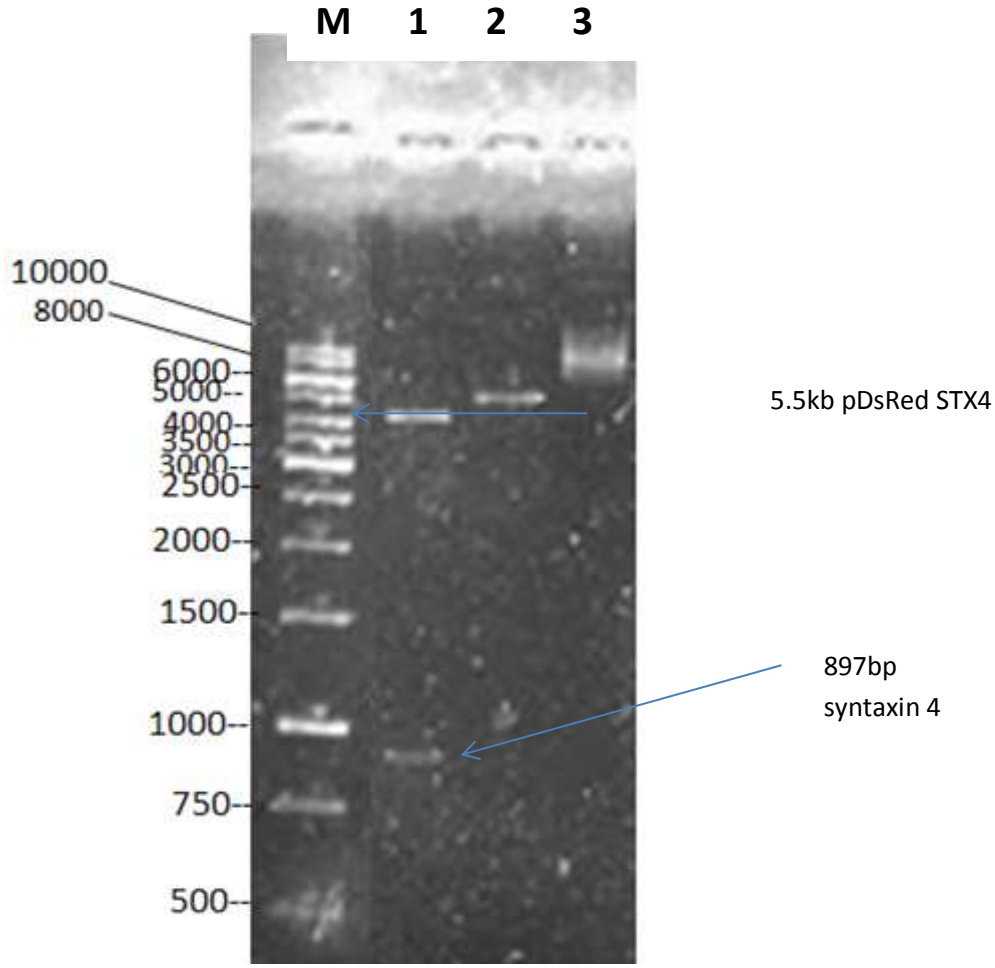


Figure 4.4.2: Agarose gel electrophoresis of single and double digested pDsRed STX 4

Lane 1: double digested pDsRed STX 4 with *EcoRI* and *Apa I. Lane 2:* Single digestion of pDsRed STX 4 plasmid with *Apa I* .*Lane* 3: uncut pDsRed STX 4. The arrow indicates fall out of syntaxin 4 which corresponds to 897 bp which corresponds with 5.5 kb with M (1kb gene ruler marker).

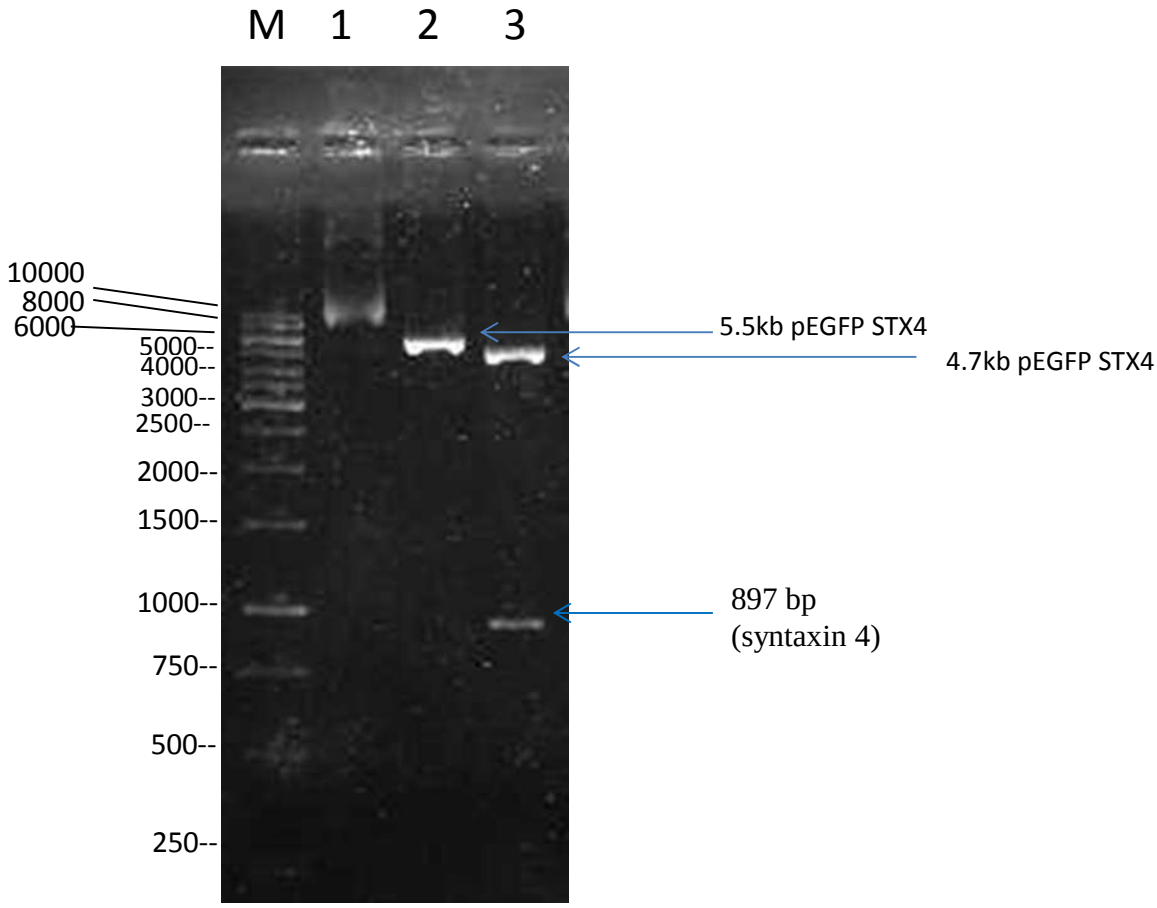


Figure 4.4.3: Agarose gel electrophoresis of single and double digested pEGFP STX 4.

Lane 1: double digested pEGFP STX 4 with *EcoRI* and *Apa I. Lane 2:* Single digestion of pEGFP STX 4 plasmid with *Apa I Lane* 3: uncut pEGFP STX 4. The arrow indicates fall out of syntaxin 4 which corresponds to 897 bp which corresponds with 5.5 kb with M (1kb gene ruler marker).

4.3 Standardization of Transfection

Here, the transfection was carried out using electroporation to increase in permeability of cell membrane. Cells were exposed to short pulses of an electric field of 950 µFarads at 320 volts resistance zero capacitance. The transfection efficiency maximized by using three different concentrations of pEGFP plasmid i.e. (5 µg, 10 µg and 20 µg of plasmid DNA) for electroporation using 5 million cells. After 24 hours post transfection the cells were harvested and analyzed both by flow cytometry and immunoflourescence microscopy for the expression of GFP and red immunofluorescence. 5µg of pEGFP plasmid transfected RBL showed 19.67%, 10 µg of pEGFP plasmid transfected RBL cells showed 37.63% and 20 µg of pEGFP transfected however 32.46% of transfection efficiency respectively (Figure 3). Transfection efficiencies were good for all the three conditions but it was highest for 10 µg pEGFP plasmid with 37.63%

transfection which has been represented in a graph (Figure 4). These slides of RBL cells transfected with 5 µg, 10 µg and 20 µg of pEGFP plasmids respectively were viewed under Nikon Eclipse Ti microscope (Figure 6) and all the images were taken at 60X magnification.

4.4 Transfection of pEGFP STX 4 and pDsRed STX 4

The maxi prep which had been previously done to increase concentration and purity of pDsRed STX 4 and pEGFP STX 4 were then used for transfection of RBL cells. 10 µg of each plasmid was taken and transfected into 5 million RBL cells. 5 million cells were transfected by electroporation at 320V and 950µF. 24 hours post transfection, the harvested cells were analyzed by flow cytometry. RBL cells showed high transfection efficiency for pEGFP STX 4 plasmid whereas transfection of pDsRed STX 4 showed lower transfection with 19.4%.

The maxi prep of pEGFP STX 4 and pEGFP STX 4 had also been used to transfect 5 million RBL cells and were cultured in coverslips. 24 hours after post transfection, cultured cells on cover slips were fixed on 4% PFA. The fixed cover slips of both pEGFP STX 4 and pDsRed STX 4 were observed under Nikon Ti Eclipse microscope. pEGFP STX 4 transfected localized on the plasma membrane whereas there was no localization of cells transfected with pDsRed STX 4. Immunoflourescence microscopy images of transfected cells with pEGFP STX 4 and pDsRed STX4 were also obtained. Figure 8 shows the immunoflourescence images for pEGFP STX 4 which can be seen on the plasma membrane whereas pDsRed STX 4 (Figure 9) doesn't show any expression of syntaxin 4 immunoflourescence on the plasma membrane.

4.4.1 Analysis of expression of pEGFP STX 4 and non-expression of pDsRed STX 4

When both pEGFP STX 4 and pDsRed STX 4 were transfected into RBL cells and observed under immunoflourescence microscopy. Only RBL cells transfected with pEGFP STX 4 localized on the plasma membrane when viewed under microscopy whereas RBL cells transfected with pDsRed STX 4 did not localize so the sequences were analyzed. The sequences for both pEGFP and pDsRed were taken from clontech and syntaxin 4 gene was taken from NCBI and compared with the sequencing results. The sequences were analyzed using sequence massager and sequence editor tools. Then the ligated sequence was translated using expasy.

pEGFP with syntaxin 4 were analyzed with sequence massager and sequence editor, then finally translated sequence were seen with expasy. The translated nucleotide sequence gave an amino acid sequence with a stop codon at the end of the translated amino acid sequence.

In case of pDsRed with syntaxin 4 also the sequence were taken and analyzed with sequence massager and sequence editor and then translated amino acid sequences were seen with expasy. For pDsRed with syntaxin 4 the nucleotide sequence gave an amino acid sequences which had stop codons in the gene with syntaxin 4. This stop codon at the middle of the nucleotide sequence is due to frame shift caused by the combination of nucleotide sequence of both pDsRed and syntaxin 4. Hence, there was no expression of pDsRed STX4 plasmid on transfected RBL cells as syntaxin 4 did not get expressed when cloned with pDsRed.

This frame shift can be removed by adding a nucleotide which helps to avoid frame shift. This can be done by site directed mutagenesis with the help of new set of primers. This primer was designed with the help of primer designing tool i.e. oligo calc.When we analyzed the new

sequence was analyzed with the new set of primers there was no frame shift and syntaxin 4 could translate on expasy with stop codon only at the end.

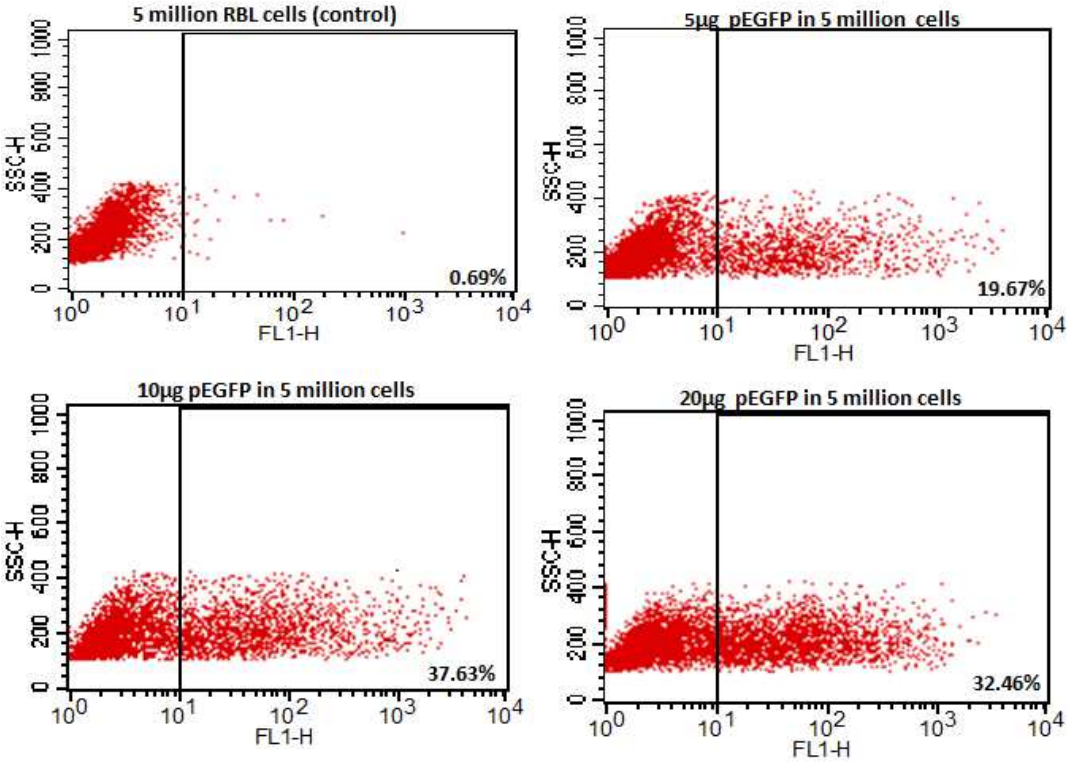


Figure 4.4.4: Standardizing transfections in RBL cells (Different DNA dose response).

5 million RBL cells were transfected with 5, 10 and 20 µg of EGFP plasmid by electroporation at 320V and 950 µF.Cells were harvested after 24 hours post-transfection and analyzed by flow cytometry.

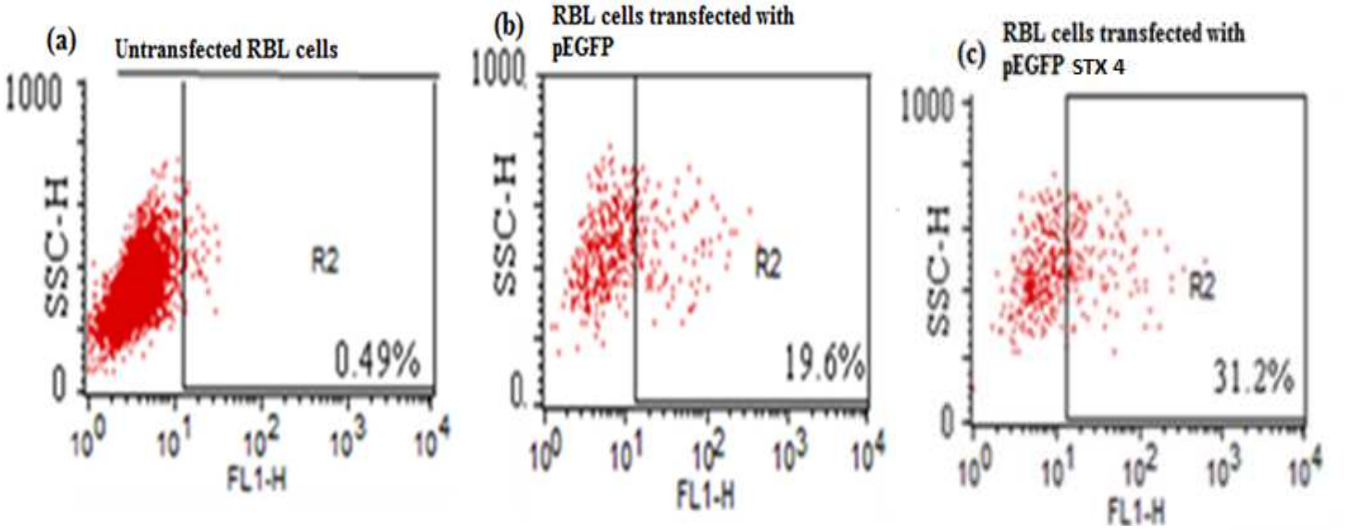


Figure 4.7Analysis of pEGFP STX 4 transfected RBL cells by flow cytometry.

5 million RBL cells were transfected with 10µg pEGFP plasmid alone and 10µg pEGFP STX 4. Cells were harvested after 24 hours and analyzed by flow cytometry. The numbers indicate the percent gated cells positive for GFP fluorescence

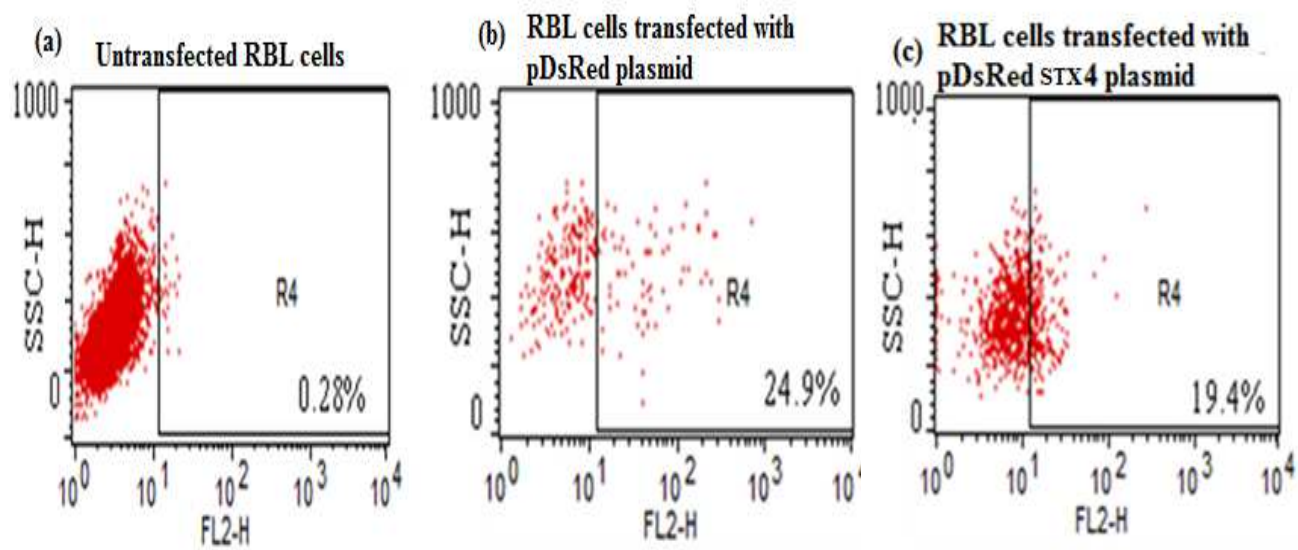


Figure 4.8: Analysis of pDsRed STX 4 transfected RBL cells by flow cytometry

5 million RBL cells were transfected with 10µg pDsRed plasmid alone and 10µg pDsRed STX 4. Cells were harvested after 24 hours and analyzed by flow cytometry. The numbers indicate the percent gated cells positive for pDsRed fluorescence.

Bioinformatics Approach: Identification of problem for non-expression of pDsRed STX4

Sequencing and translation result of pEGFP STX 4

ATGGTGAGCA AGGGCGAGGA GCTGTTCACC GGGGTGGTGC CCATCCTGGT CGAGCTGGAC
GGCGACGTAA ACGGCCACAA GTTCAGCGTG TCCGGCGAGG GCGAGGGCGA TGCCACCTAC
GGCAAGCTGA CCCTGAAGTT CATCTGCACC ACCGGCAAGC TGCCCGTGCC CTGGCCCACC
CTCGTGACCA CCCTGACCTA CGGCGTGCAG TGCTTCAGCC GCTACCCCGA CCACATGAAG
CAGCACGACT TCTTCAAGTC CGCCATGCCC GAAGGCTACG TCCAGGAGCG CACCATCTTC
TTCAAGGACG ACGGCAACTA CAAGACCCGC GCCGAGGTGA AGTTCGAGGG CGACACCCTG
GTGAACCGCA TCGAGCTGAA GGGCATCGAC TTCAAGGAGG ACGGCAACAT CCTGGGGCAC
AAGCTGGAGT ACAACTACAA CAGCCACAAC GTCTATATCA TGGCCGACAA GCAGAAGAAC
GGCATCAAGG TGAACTTCAA GATCCGCCAC AACATCGAGG ACGGCAGCGT GCAGCTCGCC
GACCACTACC AGCAGAACAC CCCCATCGGC GACGGCCCCG TGCTGCTGCC CGACAACCAC
TACCTGAGCA CCCAGTCCGC CCTGAGCAAA GACCCCAACG AGAAGCGCGA TCACATGGTC
CTGCTGGAGT TCGTGACCGC CGCCGGGATC ACTCTCGGCA TGGACGAGCT GTACAAGTCC
GGCCGGACTC AGATCTCGAG CTCAAGCTTC GAATTCATGC GCGACAGGAC CCATGAGTTG
AGGCAGGGGG ATAACATCTC AGACGATGAA GATGAGGTTC GAGTCGCGTT GGTGGTGCAC
TCAGGTGCCG CCCGGCTGAG CAGCCCGGAC GACGAGTTCT TCCAGAAGGT GCAGACAATT
CGGCAGACTA TGGCCAAACT GGAGAGTAAA GTCCGGGAGT TGGAGAAACA GCAGGTCACC
ATTCTGGCCA CGCCTCTTCC CGAGGAGAGC ATGAAGCAGG GCCTGCAGAA CCTGCGAGAG
GAGATCAAAC AGCTGGGGAG AGAAGTCCGG GCACAGCTAA AAGCCATAGA GCCCCAGAAG
GAAGAAGCTG ATGAGAATTA TAATTCAGTC AACACAAGAA TGAAGAAAAC CCAGCATGGG
GTCCTGTCCC AGCAGTTTGT GGAGCTCATC AACAAGTGTA ACTCAATGCA GTCCGAGTAC
CGAGAGAAGA ACGTGGAGCG CATTCGGCGG CAGCTGAAGA TCACCAATGC TGGAATGGTG
TCTGACGAGG AACTGGAGCA GATGCTGGAC AGTGGGCAGA GTGAGGTGTT TGTGTCTAAT
ATACTGAAGG ACACACAGGT GACCCGGCAG GCCCTGAATG AGATCTCTGC GCGACACAGT
GAGATCCAGC AGTTGGAGCG CACGATCCGT GAACTCCATG AGATCTTCAC TTTTCTAGCT
ACCGAGGTGG AGATGCAGGG AGAGATGATC AATCGTATCG AAAAGAACAT TCTGAGCTCA
GCAGACTATG TGGAACGTGG GCAAGAACAT GTCAAGATAG CGCTAGAGAA TCAGAAGAAG
GCGAGGAAGA AAAAGGTCAT GATTGCCATC TGTGTTTCTG TCACTGTTCT CATCTTGGCT
GTCATCATTG GCATCACCAT AACCGTTGGA TAAGGGCCC

After translation

Met V S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G
K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D H **Met** K Q H D
F F K S A **Met** P E G Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T L V N R I E
L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I **Met** A D K Q K N G I K V N F K
I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K
D P N E K R D H **Met** V L L E F V T A A G I T L G **Met** D E L Y K S G R T Q I S S S S F E
F **Met** R D R T H E L R Q G D N I S D D E D E V R V A L V V H S G A A R L S S P D D E F
F Q K V Q T I R Q T **Met** A K L E S K V R E L E K Q Q V T I L A T P L P E E S **Met** K Q G
L Q N L R E E I K Q L G R E V R A Q L K A I E P Q K E E A D E N Y N S V N T R **Met** K K
T Q H G V L S Q Q F V E L I N K C N S **Met** Q S E Y R E K N V E R I R R Q L K I T N A
G **Met** V S D E E L E Q **Met** L D S G Q S E V F V S N I L K D T Q V T R Q A L N E I S A R
H S E I Q Q L E R T I R E L H E I F T F L A T E V E **Met** Q G E **Met** I N R I E K N I L S S A
D Y V E R G Q E H V K I A L E N Q K K A R K K K V **Met** I A I C V S V T V L I L A V I I
G I T I T V G **Stop**

Sequencing and translation result of pDsRed STX 4

ATGGTGCGCT CCTCCAAGAA CGTCATCAAG GAGTTCATGC GCTTCAAGGT GCGCATGGAG
GGCACCGTGA ACGGCCACGA GTTCGAGATC GAGGGCGAGG GCGAGGGCCG CCCCTACGAG
GGCCACAACA CCGTGAAGCT GAAGGTGACC AAGGGCGGCC CCCTGCCCTT CGCCTGGGAC
ATCCTGTCCC CCCAGTTCCA GTACGGCTCC AAGGTGTACG TGAAGCACCC CGCCGACATC
CCCGACTACA AGAAGCTGTC CTTCCCCGAG GGCTTCAAGT GGGAGCGCGT GATGAACTTC
GAGGACGGCG GCGTGGTGAC CGTGACCCAG GACTCCTCCC TGCAGGACGG CTGCTTCATC
TACAAGGTGA AGTTCATCGG CGTGAACTTC CCCTCCGACG GCCCCGTAAT GCAGAAGAAG
ACCATGGGCT GGGAGGCCTC CACCGAGCGC CTGTACCCCC GCGACGGCGT GCTGAAGGGC
GAGATCCACA AGGCCCTGAA GCTGAAGGAC GGCGGCCACT ACCTGGTGGA GTTCAAGTCC
ATCTACATGG CCAAGAAGCC CGTGCAGCTG CCCGGCTACT ACTACGTGGA CTCCAAGCTG
GACATCACCT CCCACAACGA GGACTACACC ATCGTGGAGC AGTACGAGCG CACCGAGGGC
CGCCACCACC TGTTCCTGCT CAGATCTCGA GCTCAAGCTT CGAATTCATG CGCGACAGGA
CCCATGAGTT GAGGCAGGGG GATAACATCT CAGACGATGA AGATGAGGTT CGAGTCGCGT
TGGTGGTGCA CTCAGGTGCC GCCCGGCTGA GCAGCCCGGA CGACGAGTTC TTCCAGAAGG
TGCAGACAAT TCGGCAGACT ATGGCCAAAC TGGAGAGTAA AGTCCGGGAG TTGGAGAAAC
AGCAGGTCAC CATTCTGGCC ACGCCTCTTC CCGAGGAGAG CATGAAGCAG GGCCTGCAGA
ACCTGCGAGA GGAGATCAAA CAGCTGGGGA GAGAAGTCCG GGCACAGCTA AAAGCCATAG
AGCCCCAGAA GGAAGAAGCT GATGAGAATT ATAATTCAGT CAACACAAGA ATGAAGAAAA
CCCAGCATGG GGTCCTGTCC CAGCAGTTTG TGGAGCTCAT CAACAAGTGT AACTCAATGC
AGTCCGAGTA CCGAGAGAAG AACGTGGAGC GCATTCGGCG GCAGCTGAAG ATCACCAATG
CTGGAATGGT GTCTGACGAG GAACTGGAGC AGATGCTGGA CAGTGGGCAG AGTGAGGTGT
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CGCGACACAG TGAGATCCAG CAGTTGGAGC GCACGATCCG TGAACTCCAT GAGATCTTCA
CTTTTCTAGC TACCGAGGTG GAGATGCAGG GAGAGATGAT CAATCGTATC GAAAAGAACA
TTCTGAGCTC AGCAGACTAT GTGGAACGTG GGCAAGAACA TGTCAAGATA GCGCTAGAGA
ATCAGAAGAA GGCGAGGAAG AAAAAGGTCA TGATTGCCAT CTGTGTTTCT GTCACTGTTC
TCATCTTGGC TGTCATCATT GGCATCACCA TAACCGTTGG ATAAGGGCCC

After translation

Met V R S S K N V I K E F **Met** R F K V R **Met** E G T V N G H E F E I E G E G E G R P Y E
G H N T V K L K V T K G G P L P F A W D I L S P Q F Q Y G S K V Y V K H P A D I P D Y
K K L S F P E G F K W E R V **Met** N F E D G G V V T V T Q D S S L Q D G C F I Y K V K
F I G V N F P S D G P V **Met** Q K K T **Met** G W E A S T E R L Y P R D G V L K G E I H K A
L K L K D G G H Y L V E F K S I Y **Met** A K K P V Q L P G Y Y Y V D S K L D I T S H N E
D Y T I V E Q Y E R T E G R H H L F L L R S R A Q A S N S C A T G P **Met** S **Stop** G R G I
T S Q T **Met** K **Met** R F E S R W W C T Q V P P G **Stop** A A R T T S S S R R C R Q F G R L
W P N W R V K S G S W R N S R S P F W P R L F P R R A **Stop** S R A C R T C E R R S N S
W G E K S G H S **Stop** K P **Stop** S P R R K K L **Met** R I I I Q S T Q E **Stop** R K P S **Met** G S
C P S S L W S S S T S V T Q C S P S T E R R T W S A F G G S **Stop** R S P **Met** L E W C L T
R N W S R C W T V G R V R C L C L I Y **Stop** R T H R **Stop** P G R P **Stop** **Met** R S L R D T
V R S S S W S A R S V N S **Met**R S S L F **Stop** L P R W R C R E R **Stop** S I V S K R T
F **Stop** A Q Q T **Met** W N V G K N **Met** S R **Stop** R **Stop** R I R R R R G R K R S **Stop** L P S V
F L S L F S S W L S S L A S P **Stop** P L D K G

Primer to remove frame shift of pDsRed STX 4 for site directed mutagenesis

Forward primer

5’CGAGCTCAAGCTTCGAATTCTATGCGCGACAGGA 3’

Reversed primer

3’ TCCTGTCGCGCATATATTTCTATGCTTGAGCTCG 5’

pDsRed STX 4 sequence after site directed mutagenesis with new primer

ATGGTGCGCT CCTCCAAGAA CGTCATCAAG GAGTTCATGC GCTTCAAGGT GCGCATGGAG
GGCACCGTGA ACGGCCACGA GTTCGAGATC GAGGGCGAGG GCGAGGGCCG CCCCTACGAG
GGCCACAACA CCGTGAAGCT GAAGGTGACC AAGGGCGGCC CCCTGCCCTT CGCCTGGGAC
ATCCTGTCCC CCCAGTTCCA GTACGGCTCC AAGGTGTACG TGAAGCACCC CGCCGACATC
CCCGACTACA AGAAGCTGTC CTTCCCCGAG GGCTTCAAGT GGGAGCGCGT GATGAACTTC
GAGGACGGCG GCGTGGTGAC CGTGACCCAG GACTCCTCCC TGCAGGACGG CTGCTTCATC
TACAAGGTGA AGTTCATCGG CGTGAACTTC CCCTCCGACG GCCCCGTAAT GCAGAAGAAG
ACCATGGGCT GGGAGGCCTC CACCGAGCGC CTGTACCCCC GCGACGGCGT GCTGAAGGGC
GAGATCCACA AGGCCCTGAA GCTGAAGGAC GGCGGCCACT ACCTGGTGGA GTTCAAGTCC
ATCTACATGG CCAAGAAGCC CGTGCAGCTG CCCGGCTACT ACTACGTGGA CTCCAAGCTG
GACATCACCT CCCACAACGA GGACTACACC ATCGTGGAGC AGTACGAGCG CACCGAGGGC
CGCCACCACC TGTTCCTGCT CAGATCTCGA GCTCAAGCTT CGAATTCTAT GCGCGACAGG
ACCCATGAGT TGAGGCAGGG GGATAACATC TCAGACGATG AAGATGAGGT TCGAGTCGCG
TTGGTGGTGC ACTCAGGTGC CGCCCGGCTG AGCAGCCCGG ACGACGAGTT CTTCCAGAAG
GTGCAGACAA TTCGGCAGAC TATGGCCAAA CTGGAGAGTA AAGTCCGGGA GTTGGAGAAA
CAGCAGGTCA CCATTCTGGC CACGCCTCTT CCCGAGGAGA GCATGAAGCA GGGCCTGCAG
AACCTGCGAG AGGAGATCAA ACAGCTGGGG AGAGAAGTCC GGGCACAGCT AAAAGCCATA
GAGCCCCAGA AGGAAGAAGC TGATGAGAAT TATAATTCAG TCAACACAAG AATGAAGAAA
ACCCAGCATG GGGTCCTGTC CCAGCAGTTT GTGGAGCTCA TCAACAAGTG TAACTCAATG
CAGTCCGAGT ACCGAGAGAA GAACGTGGAG CGCATTCGGC GGCAGCTGAA GATCACCAAT
GCTGGAATGG TGTCTGACGA GGAACTGGAG CAGATGCTGG ACAGTGGGCA GAGTGAGGTG
TTTGTGTCTA ATATACTGAA GGACACACAG GTGACCCGGC AGGCCCTGAA TGAGATCTCT
GCGCGACACA GTGAGATCCA GCAGTTGGAG CGCACGATCC GTGAACTCCA TGAGATCTTC
ACTTTTCTAG CTACCGAGGT GGAGATGCAG GGAGAGATGA TCAATCGTAT CGAAAAGAAC
ATTCTGAGCT CAGCAGACTA TGTGGAACGT GGGCAAGAAC ATGTCAAGAT AGCGCTAGAG
AATCAGAAGA AGGCGAGGAA GAAAAAGGTC ATGATTGCCA TCTGTGTTTC TGTCACTGTT
CTCATCTTGG CTGTCATCAT TGGCATCACC ATAACCGTTG GATAAGGGCC C

pDsRed STX 4 sequence after site directed mutagenesis with new primer after translation

Met V R S S K N V I K E F **Met** R F K V R **Met** E G T V N G H E F E I E G E
G E G R P Y E G H N T V K L K V T K G G P L P F A W D I L S P Q F Q Y G
S K V Y V K H P A D I P D Y K K L S F P E G F K W E R V **Met** N F E D G G
V V T V T Q D S S L Q D G C F I Y K V K F I G V N F P S D G P V **Met** Q K K
T **Met** G W E A S T E R L Y P R D G V L K G E I H K A L K L K D G G H Y L
V E F K S I Y **Met** A K K P V Q L P G Y Y Y V D S K L D I T S H N E D Y T I
V E Q Y E R T E G R H H L F L L R S R A Q A S N S **Met**R D R T H E L R Q
G D N I S D D E D E V R V A L V V H S G A A R L S S P D D E F F Q K V Q
T I R Q T **Met** A K L E S K V R E L E K Q Q V T I L A T P L P E E S **Met** K Q
G L Q N L R E E I K Q L G R E V R A Q L K A I E P Q K E E A D E N Y N S V
N T R **Met** K K T Q H G V L S Q Q F V E L I N K C N S **Met** Q S E Y R E K N
V E R I R R Q L K I T N A G **Met** V S D E E L E Q **Met** L D S G Q S E V F V S
N I L K D T Q V T R Q A L N E I S A R H S E I Q Q L E R T I R E L H E I F T
F L A T E V E **Met** Q G E **Met** I N R I E K N I L S S A D Y V E R G Q E H V K

4.7 Cloning of SNAP 25

RNA was isolated from 5 million PC 12 cells using TRI reagent. The concentration of RNA was was seen by nanodrop 2000 and the concentration was found to be 1333.1ng/µl. The isolated RNA was reverse transcribed into cDNA with oligo dT primer using M-MuLV Reverse transcriptase in Reverse Transcriptase PCR. In order to check the quality of cDNA the amplification of the cDNA by conventional PCR for GAPDH using rat GAPDH specific primer was done using Taq polymerase. Then for cloning of SNAP 25 into pEGFP vector, SNAP 25 was amplified using SNAP 25 specific primers with pfu polymerase in conventional PCR. PCR purified SNAP-25 and vector pEGFP-C2 were double digested with enzymes *EcoRI* and *BamHI*. Double digested SNAP-25 and pEGFP-C2 were then run on 0.8% agarose gel. The DNA bands of double digested SNAP 25 (insert) and pEGFP-C2 (vector) were excised out in UV illuminator. The gel was then purified by Qiagen Gel Purification kit. The purified SNAP-25 and pEGFP-C2 were ligated with T4 DNA Ligase after incubating overnight at 16 ℃. The ligated products were transformed into *E.coli* DH5α cells. The colonies thus obtained were checked for the gene of interest by colony PCR and confirmed by double digestion of isolated plasmid with *BamHI* and *EcoRI* for the required fall out of SNAP 25 with 635 bp. The restrictiondigested plasmid with the highest expression was sent for sequencing and the concentration of the isolated plasmid was increased by maxi prep. Then, the isolated plasmids expression was seen in RBL mast cell lines by immunoflourescence microscopy.

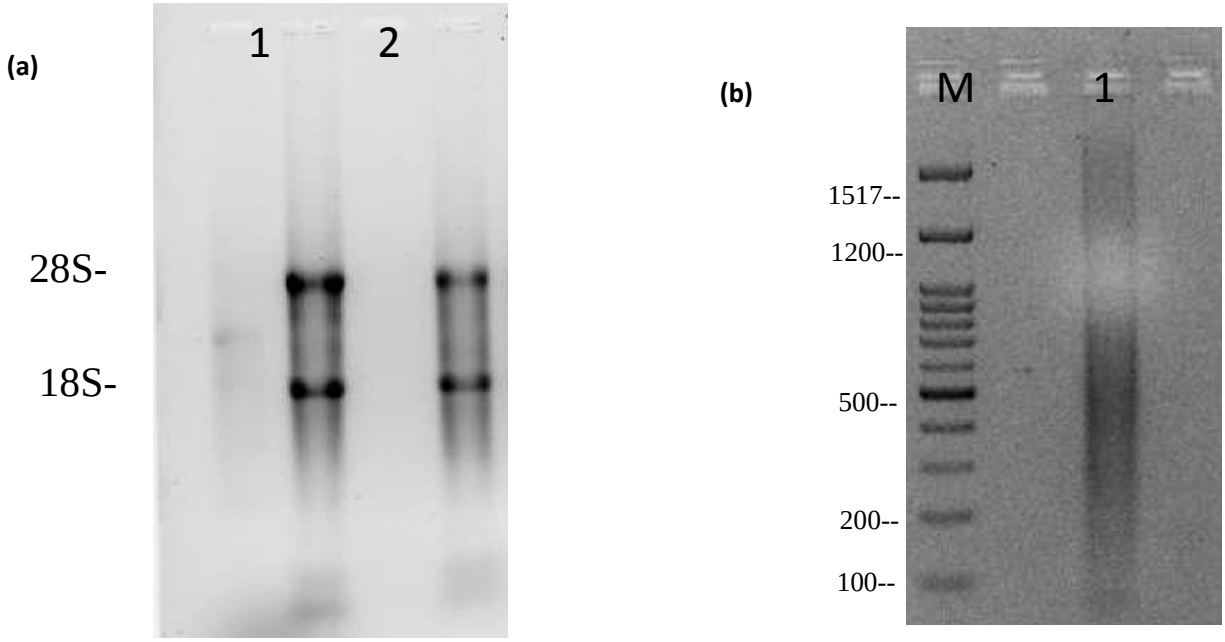

Figure 4.13: Formaldehyde agarose gel electrophoresis of RNA extracted from PC12 cell line (1.2% Formaldehyde agarose)

a: RNA gel showed two distinct bands of 28s and 18s along with control p815 RNA Lane 1:PC-12 RNA (3µg),Lane 2:positive control RNA,P815(3 µg)

b: Agarose gel electrophoresis of c-DNA of PC-12 cell line. cDNA of PC12 was run on 1.5% agarose gel where M contains 100 bp DNA ladder(NEB)(0.5µg), lane 1: PC12 cDNA

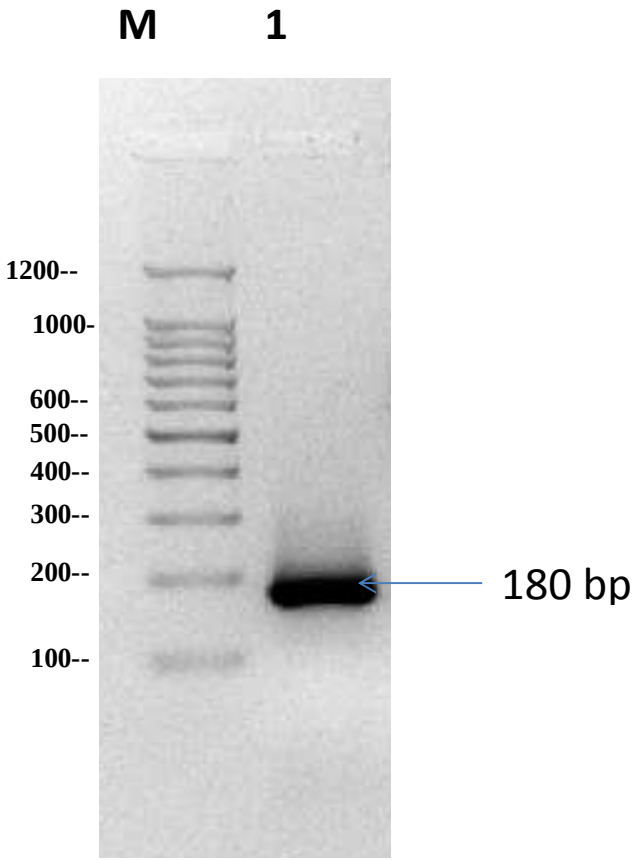

Figure 4.14: Agarose gel electrophoresis of rat GAPDH.

PCR of GAPDH was run on 1.8% agarose gel Lane1: GAPDH showed a clear band of 180 bp which corresponds to M (100 bp marker)

cDNA of rat was checked by running PCR of GAPDH with rat GAPDH primer. The PCR product was run on 1.8% agarose. A clear band of 180bp was seen on the gel therefore this cDNA was further used to amplify SNAP 25 gene.

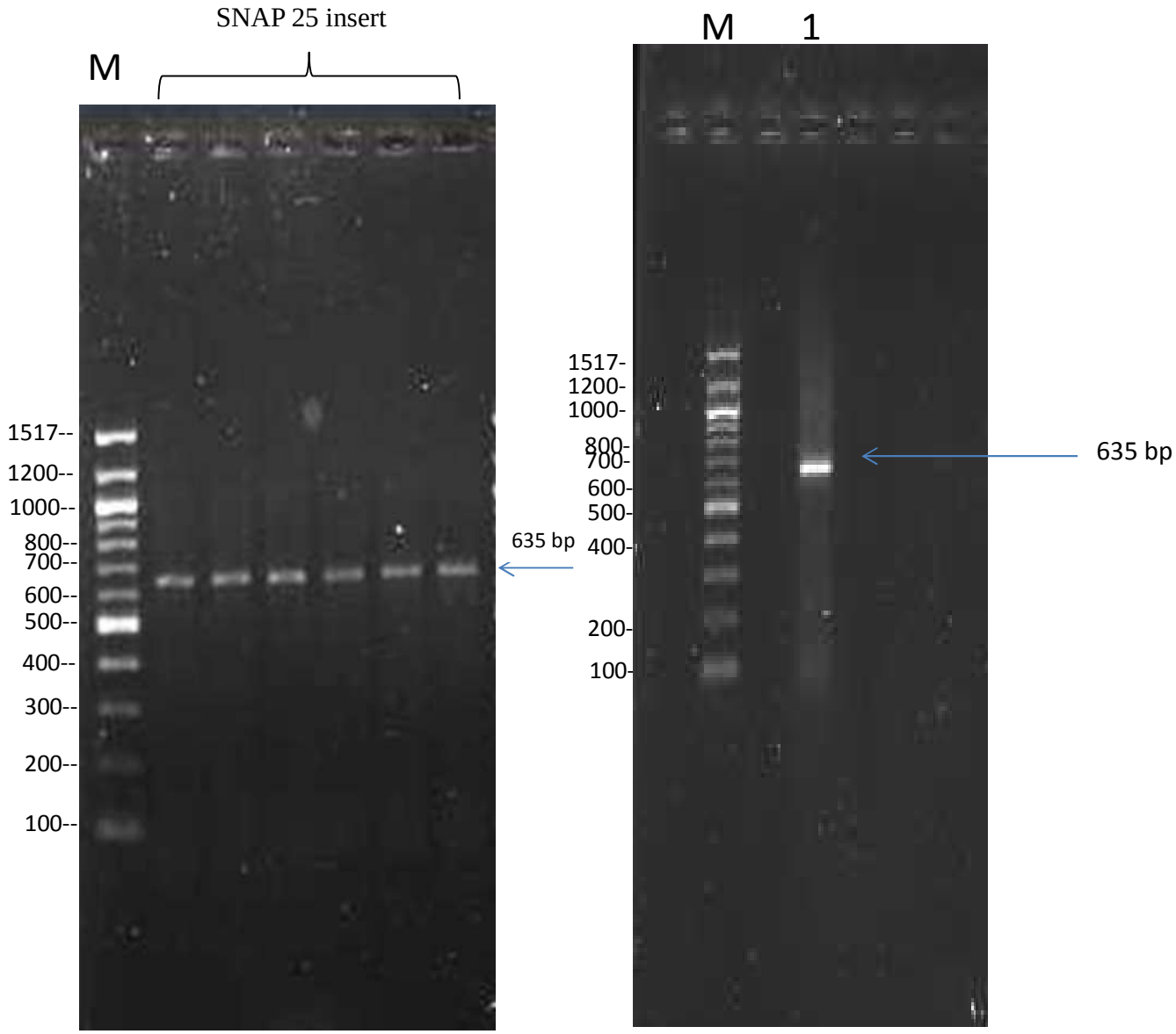

Figure 4.15 a: Agarose gel electrophoresis of rat SNAP 25 : Six PCR product of SNAP 25 was run on 1.8% agarose and all the PCR product gave a clear band of 635bp which corresponds to M (100 bp ladder).

b: Agarose gel of PCR purified SNAP 25: PCR purification of SNAP 25 of 635 was run on 1.8% agarose gel which corresponds to M (100 bp ladder).

PCR of cDNA of rat was run for SNAP 25 gene with SNAP 25 primer containing *EcoRI* and *BamHI* restriction site. The PCR sample were run on 1.8% agarose gel and a clear band of 635 bp was seen. All six PCR products of SNAP 25 were combined together and PCR product waspurified by PCR purification kit (QIAGEN).

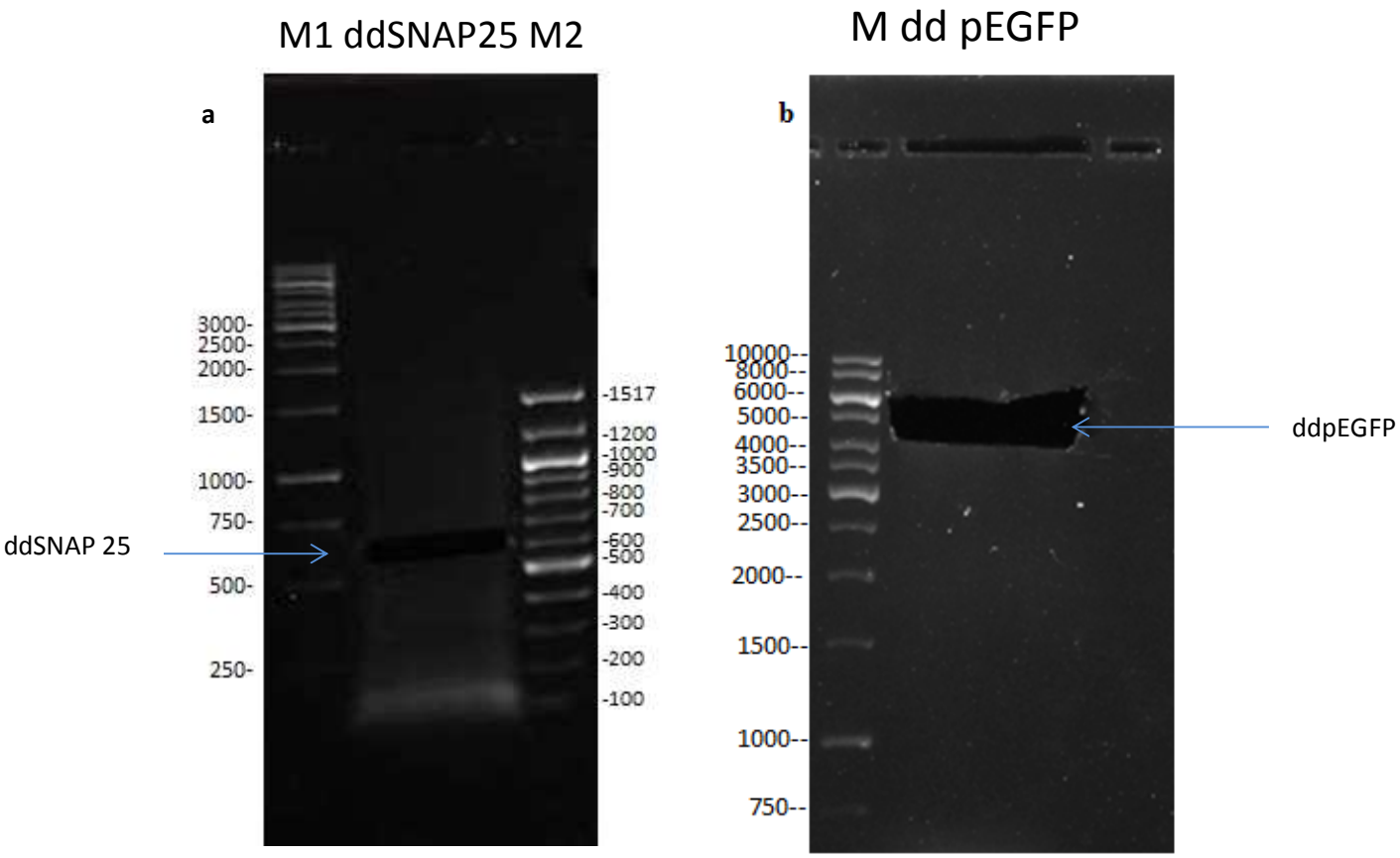

Figure4.16: Gel elution of double digested SNAP 25 and double digested pEGFP.

Double digested SNAP 25 and pEGFP was eluted form on 0.8% agarose gel. (a) Shows double digested and eluted SNAP 25 of 635bp which corresponds to M1 (1 kb ladder) and M2 (100bp ladder).

PCR purified SNAP 25b was double digested with *EcoRI* and *BamHI* and run in gel. Then SNAP 25 was eluted from gel.

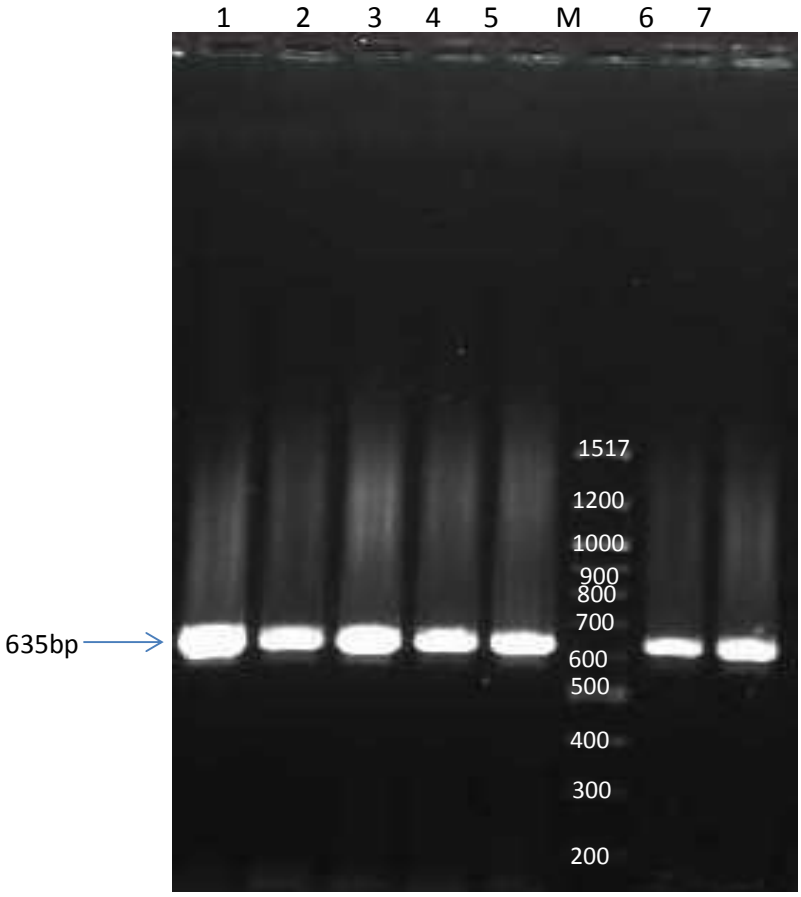

Figure 4.17: Agarose gel electrophoresis of PCR of positive colonies: PCR Product of positive colonies was run on 1.8% agarose

Lane 1:Colony PCR of colony 1,Lane 2:Colony PCR of colony 2,Lane 3:Colony PCR of colony 3,Lane 4:Colony PCR of colony 4,Lane 5:Colony PCR of colony 5,Lane 6:Colony PCR of colony 6 and Lane 7: Colony PCR of colony 7. The colony PCR of all the colonies gave SNAP 25 of 635bp which corresponds to M (1kb ladder)

The positive colonies from kanamycin positive plate after ligation was tested by colony PCR.

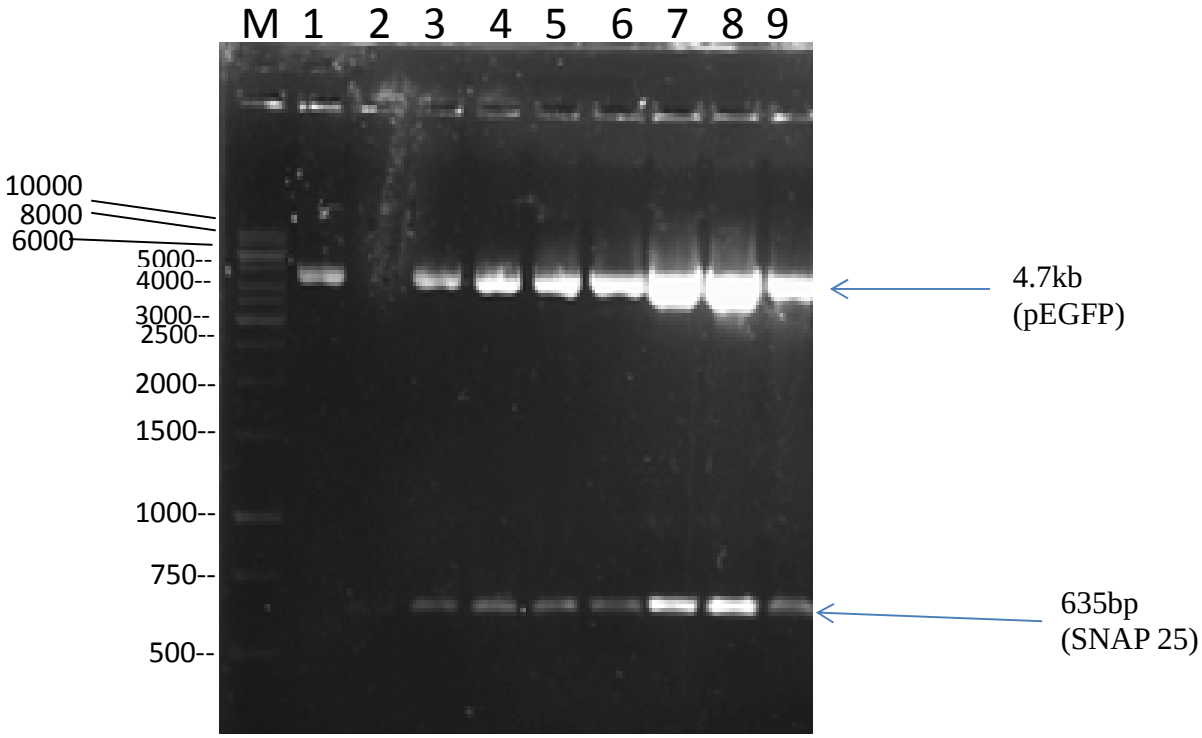

Figure 4.18: Agarose gel electrophoresis of double digested plasmid isolated form PCR positive colonies

Lane1: Single digested pEGFP with *BamHI*, Lane 2: Double digested SNAP 25, Lane 3:DD of Plasmid(colony1), Lane 4:DD of Plasmid(colony2), Lane 5:DD of Plasmid(colony3), Lane 6:DD of Plasmid(colony4), Lane 7:DD of Plasmid(colony5), Lane 8:DD of Plasmid(colony6), Lane 9:DD of Plasmid(colony7) where the fall out of all the plasmid of positive colonies gave a band of 635 bp of SNAP 25 which corresponds to M (1kb ladder).

The plasmids of colonies positive on colony PCR were isolated by alkaline lysis and the colonies were further confirmed with double digestion with *EcoRI* and *BamHI*. Among the seven plasmids isolated seven had fallout of 635bp of SNAP 25.These confirmed our positive clone with pEGFP of 4.7kb with 635bp SNAP 25 insert

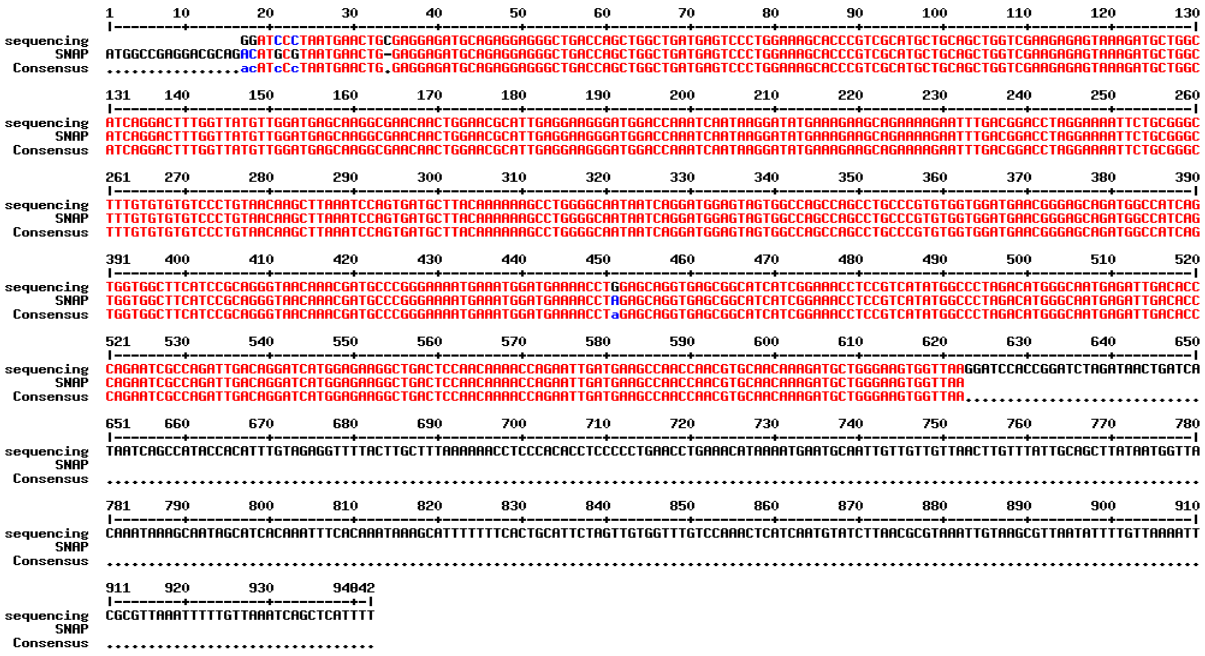

Figure 4.19: Multiple alignment of SNAP 25 sequence with sequencing result from forward primer

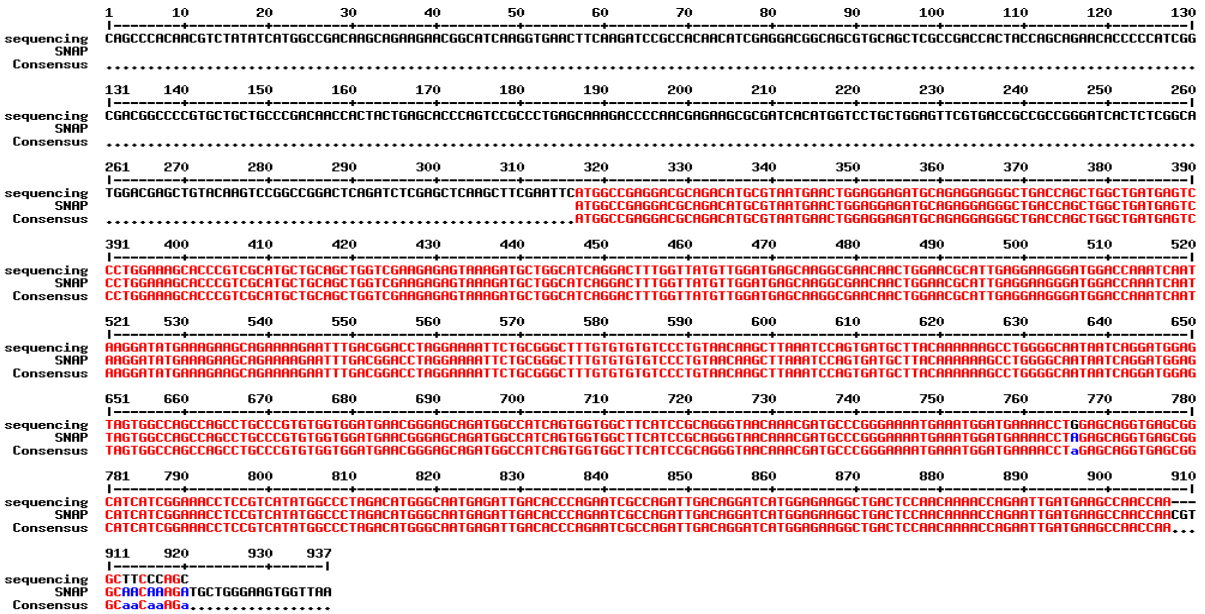

Figure 4.20: Multiple alignment of SNAP 25 sequence with sequencing result from reverse primer.

CHAPTER 5 Discussion

Mast cells are leukocytes that circulate in the blood in an immature form before migrating to vascularized tissues, and are finally located in close contact with the external environment, such as skin, airways, and intestines. They participate in the early recognition of pathogens within seconds of stimulation, mast cells can undergo degranulation, rapidly releasing pre-formed mediators present within cytoplasmic granules, including histamine, the proteases tryptase and chymase, and pre-formed tumor necrosis factor alpha (Urb *et al., 2*012). Previously Mast cells were thought only to promote inflammation and other tissue changes in IgE associated allergic disorders but recent studies have shown that mast cells can function as effector cells during innate and adaptive, immune responses. Secretion of factors such as tumor-necrosis factor (TNF), chemokines, histamine, LTB 4 and proteases promote the migration, maturation, differentiation and function of other immune cells. Secretion of interleukin-4 (IL-4), IL-13 and CD40L promote the production of IgE by B cells promote the production of chemokines and cytokines by airway smooth muscle cells (through the secretion of TNF, IL-4 and IL-13 whereas the secretion of IL-10 suppresses cytokine production by T cell and the production of pro-inflammatory cytokines and chemokines (Galli *et al*., 2008). The key proteins involved in the docking and fusion of vesicles for degranulation are SNARE proteins.

A typical mast cells trans-SNARE complex at the plasma membrane includes a vesicular SNARE (v-SNARE) such as vesicle associated membrane protein (VAMP) that pairs with two target membrane SNAREs (t-SNAREs) such as a Syntaxin (STX) molecule and synaptosome associated protein of 23 (ubiquitous) containing two SNARE motifs. The role of SNARE proteins in the release of *de novo* synthesized mediators like cytokines or chemokines from mast cells has hardly been analyzed. The data so far indicate that chemokine/cytokine secretion does not follow a unique pathway but rather represents a complex machinery of multiple pathways and organelles (Lorentz *et al*., 2012). Though the study of many SNARE proteins is ongoing but still a lot is left to be uncovered. RBL-2H3 cells, like mast cells respond with degranulation following crosslinking of their IgE-bound FceRI by multivalent allergens, with the release of a range of preformed and newly synthesized mediators that evoke a potent response and serve as an established model system to study the mast cell functions in vitro (Passante *et al*., 2009).

In this study, the expression of syntaxin 4 was seen by Western Blot by using anti syntaxin 4 rabbit polyclonal antibody. Western Blot analysis of RBL cells, mice brain and spleen homogenate. The western blot analysis show the presence of t-SNARE syntaxin 4 in RBL cells and mice spleen homogenate at around 34 kDa and faint expression of syntaxin 4 in mice brain as in neurons, vesicles t-SNAREs syntaxin 1a and SNAP-25 on the presynaptic membrane, catalyzes membrane fusion for exocytosis (Jahn *et al., 2*006). The study of any particular protein can be done in vitro by knock down of that gene or by over expression of that same gene. Over expression of a particular protein in RBL-2H3 cells can be done by transfecting the particular gene by electroporation. A high efficiency transient transfection protocol has been previously described (Puri *et al*. 2003), replication of the protocol was done in order to maximize transfection efficiency where 10µg of DNA with 5 million RBL cells showed maximum of 37.63%.

Pharmacological approaches or those based on the use of antibodies to deplete mast cells or to neutralize their products can also provide useful information, but they are limited by the specificity of the drug or antibody chosen. For example, antihistamines block the actions of histamine whether it has been secreted by mast cells or by non-mast cells, and antibodies that neutralize SCF36 or block KIT37, 38 can result in the depletion of mast cells *in vivo*, but can also influence other cell types that express KIT (Galli *et al., 2*008). By studying the molecular mechanism of the proteins involved in the degranulation of different chemokines, cytokines we can formulate a better way for therapeutics that will be more precise and effective.

The molecular mechanisms of these SNARE proteins can be studied in vitro by cloning it different vectors. The GFP and pDsRed tagged syntaxin 4 protein will help in various studies like localization, by actual visualization its responses to various triggers in vitro. These can be done with the help of immunoflourescence microscopy, flow cytometry and live cell imaging. Transfected RBL cells with GFP tagged syntaxin 4 and pDsRed tagged syntaxin 4 showed high percentage of transfection with 31.2% with GFP tagged with syntaxin 4 and 19.4% with DsRed tagged syntaxin 4 by flow cytometry analysis. The transfected cells show high level of transfection but to study the expression of syntaxin 4, immunoflourescence microscopy was done. The transfection of syntaxin 4 tagged with GFP vector (pEGFP STX 4) showed localization in plasma membrane after 24 hours, whereas transfection of syntaxin 4 with pDsRed vector showed very less expression of red immunoflourescence and no localization on the plasma membrane.

During translation of genetic code, the mRNA is read sequentially in set of three nucleotides. Each nucleotide triplet or a codon, specifies the amino acid which is added to the growing peptide chain. The reading frame of the codon is established at the initiation of peptide synthesis and is maintained by the mechanism which moves the ribosomes along the message three nucleotides at a time. Frame shift mutations are addition or deletions of nucleotide which shift the reading frame creating an entirely new set of codon beyond the mutation (Riddle *et al*., 1973). The understanding of molecular mechanism of gene was analyzed to understand the negligible red fluorescence of syntaxin 4 tagged with pDsRed. The sequence of the cloned syntaxin 4 revealed a frame shift mutation in syntaxin 4 protein as described by Riddle *et al*. The frame shift in pDsRed created an entire new set of codon after the restriction site of *E.coRI* which brought a stop codon in the beginning of syntaxin 4. Hence, no red immunoflourescence could be seen localizing on the plasma membrane. Since in the fusion protein pDsRed is present at N- terminal, it transcribed and successfully translated giving red fluorescence. Whereas the sequence of pEGFP STX 4 showed no such frame shift mutation and expressed well on transfected RBL cells. This sequence was analyzed and to rectify it, one nucleotide must be added in between pDsRed and syntaxin 4 which will not change the sequence of both pDsRed and syntaxin 4 and both pDsRed tagged syntaxin will be translated. A primer was designed for site directed mutagenesis to include an extra nucleotide which will remove the frame shift mutation.

The tagging of syntaxin 4 in both green immunoflourescence and red immunoflourescence is useful for studies in vitro. With different color tagged syntaxin 4 in preliminary studies helps us study the colocalization, interaction with different proteins, antibodies, different SNARE

proteins and SNARE interacting proteins in future experiments. This would bring more flexibility in *in vitro* studies and more exploration can be done.

Syntaxin 4 shows a ubiquitous tissue distribution, functions in pathways such as the exocytosis of vesicles containing the facilitative glucose transporter, Glut4, in adipocytes and skeletal muscle. Syntaxin 4 in particular is a Qa SNARE that has been shown to play an important role in regulated secretion on the plasma membrane of neutrophils, macrophage, eosinophils and STX4 is necessarily involved in the secretion process of Ig by human plasma cells (Rahman *et al., 2*013; Murray *et al.,* 2006 and Jaramillo *et al* 2013). It has been noted that in human mast cells STX3, like STX4, is localized at the plasma membrane, while in rodent mast cells it is found mainly on secretory granules (Lorentz *et al., 2*012). In human mast cells chemokine release could be mediated by a Q-SNARE complex consisting of SNAP-23, STX3, or STX4 and the R-SNARE, VAMP7 or VAMP8 at the plasma membrane (Sander *et al*., 2008). Membrane association kinetics of RBL cells showed us that after fixation of transfected cells at different time intervals, the pEGFP STX 4 expression was seen at minimum of 3 hours after transfection and it also started to localize to the plasma membrane and finally at 24 hours it showed almost prominent localization on plasma membrane. The plasma membrane localization of pEGFP STX 4 was confirmed by complete colocalization with DiIC 16, a lipid dye known to bind plasma membrane lipids in cells (Baumgart *et al., 2*009). The transfected RBL cells with pEGFP STX plasmid showed that syntaxin 4 colocalizes with the plasma membrane after 24 hours. The microscopy and co-localization analysis gives a precise and clear idea of its localization. Further studies with the pEGFP STX 4 clone can be done to know its function and its role in mast cells effector function as well as in other immune cells has to done to explore more on t-SNARE STX

Synaptosomal-associated protein of 25 kDa (SNAP-25) is a membrane protein expressed mostly in neurons and endocrine cells plays an essential role in neurotransmitter release through its association with other SNARE proteins, synaptobrevin and syntaxin 1. Rat phaeochromocytoma (PC12) cells resemble the phenotype of sympathetic ganglion neurons upon differentiation with nerve growth factor (NGF) which can be used as an alternative model to investigate exocytosis for neurosecretory, neurobiology and neurochemistry studies. PC12 cell is mainly because of their extreme versatility for pharmacological manipulation, their ease of culture and the large amount of background knowledge on their proliferation and differentiation. Moreover, they more closely resemble neurons with smaller vesicle and quantal size than chromaffin cells (Westerink *et al* 2008, Greene *et al*, 1976). SNAP 25 gene was fused in pEGFP vector for various studies like function, localization and also for its expression on non-neuronal cells. Both murine and human mast cells have SNAP 23 as its representative of Qb,c family (Woska *et al*., 2012) but Salinas *et al., 2*004 has shown the presence of SNAP 25 rat peritoneal mast cells with immunoblot assay and imunoflourescence microscopy. In our study, SNAP 25 gene was taken from PC12 cells and fused in pEGFP vector. This pEGFP fused SNAP 25 was further sent for sequencing.Sequencing results showed one mutation when compared to the sequence given in NCBI. The mutation was in 451 position from A to G transition from CTA to CTG with the forward primer where the there was high peak in DNA sequence chromatogram. However when its protein translation was examined no effect was seen on the reading frame as both coded for the same amino acid Leucine.

CHAPTER 6 CONCLUSION

Mast cells are fascinating, multifunctional, bone marrow derived, tissue-dwelling cells. They play a central role in inflammatory and immediate allergic reactions and also can be important as initiators and effectors of innate immunity and adaptive immunity. Within seconds of stimulation, mast cells can undergo degranulation, rapidly releasing pre-formed mediators present within cytoplasmic granules, including histamine, the proteases tryptase and chymase, and pre-formed tumor necrosis factor α. Mast cells also require SNARE proteins for the membrane fusion (Abraham *et al.*, 2010**,** Galli *et al.*, 2006**).** The preliminary data in this study details the expression and localization of t-SNARE in RBL cells. The expression of syntaxin 4 was seen by immunoblotting and localization was seen by green flourescence tagged syntaxin 4 in RBL cells as mast cell model. Replication of standardization of transfection was done where maximum transfection efficeincy was seen to be 37.63% with 5 million RBL cells with 10µg pEGFP plasmid. The problem for negligible red flourescence for pDsRed stx 4 was identified and primer was designed to rectify the problem. The location of syntaxin 4 in green flourescence protein was confirmed by co-staining with DiIC 16 stain.SNAP 25 gene was isolated from PC 12 gene and fused in pEGFP vector and its expression was seen on RBL cells with immunoflourescence microscopy.

Mast cell degranulation is a critical step in the immune system which can bring about both positive effects in innate and adaptive immunity and can also cause inflammation in allergic reactions. The trigger for different degranulations of different cytokines,chemokines have specific set of SNARE proteins involved in it. By studing the molecular mechanism, its location and expression we can use it for therapeutic purposes. As shown by this study, pEGFP stx 4's cloned plasmid can be fruther used to study interaction of syntain 4 with different other SNAREs or SNARE interacting proteins, its function in different other immune cells can be studied.

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APPENDICES

PBS (Phosphate Buffer Saline): 1 litre (pH maintained at 7.3-7.4)

8gm NaCl

1.44gm $NaHPO_4.2H_2O$

0.2gm KCl

0.2 gm KH_2PO_4

For plasmid DNA isolation

STE Solution

- 0.1M NaCl
- 10 mM Tris-Cl(pH 8)
- 1mM EDTA(pH-8)

Solution I

- 50 mM Glucose
- 25 mM Tris Cl(pH-8)
- 10 mM EDTA(pH-8)

Solution II(freshly prepared)

- 0.2 N NaOH
- 1% SDS

Solution III

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

TBE buffer(5X)-1 litre

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

6 X Gel loading dye

Bromophenol blue	0.03%(W/V)
Xylene cyanol FF	0.03%
Glycerol	60%

60 mM EDTA

10mM Tris (pH-8.0)

Immunoflourescence microscopy Reagents

4% PFA (fixative)

4 gm Paraformahdehyde

100ml PBS

Quencher

15mM NH_4Cl in PBS

Western Blot and SDS-PAGE Solutions:

2X sample Buffer

20 ml glycerol
25 ml upper gel buffer 4X
4 gram SDS
200 ul of 2% Bromophenol Blue
Make upto 100ml with MQ, then add 2% final working conc. β-ME as needed while making 1X

4X Sample Buffer:

40 ml glycerol
50 ml upper gel buffer 4X
8 gram SDS
400 ul of 2% Bromophenol Blue
Make total 100ml. Then add 2% Bromophenol Blue as needed

4X lower gel buffer (pH 8.8)

90.8 g Tris
2 g SDS
Make 500 ml.

4X upper Gel buffer (pH6.8)

30.3 g Tris
2 g SDS
Make 500 ml.

30% acrylamide:

29 gram acrylamide
1g bis-acrylamide

10X Gel Running Buffer (1 lit):

30.25 g Tris
144 g Glycine
10 g SDS
pH-8.3

Sequencing Results

pDsRedStx 4

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