BIOINFORMATIC APPROACH FOR GENERATION OF A FUSION PROTEIN OF t-SNARE SNAP-23 CONTAINING A TRANSMEMBRANE DOMAIN

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This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

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Certificate

This is to certify that the thesis report entitled **"Bioinformatic approach for Generation of a fusion protein of t-SNARE SNAP-23 containing a transmembrane domain**" is a bonafide work of **Mr. Govind Prasad Sah**, towards the completion of his dissertation in partial fulfilment for the requirement of degree of MASTER OF SCIENCE IN BIOTECHNOLOGY. This research work has been carried out at **School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.**

This research work is original and has not been submitted so far, in part or in full, for any other degree or diploma of any University.

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Certificate of Evaluation

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Date: 10 January, 2012 Govind Prasad Sah

LIST OF ABBREVIATIONS

'µg'	: microgram
'µl'	: microlitre
'mg'	: milligram
'ml'	: millilitre
ATP	: Adenosine Tri Phosphate
BoNT	: Botulinum Neurotoxin
cAMP	: Cyclic Adenosine Mono Phosphate
cGMP	: Cyclic Guanosine Mono Phosphate
CLP24	: Claudin Like Protein 24
DEPC	: Diethylpyrocarbonate
DNA	: Deoxy Ribonucleic Acid
EDTA	: Ethyl Diamine Tetra Aceticacid
ExPASy	: Expert Protein Analysis System
GPI	: Glycerophospho-inositol
IKK2	: Inhibitor of nuclear factor kappa-B kinase subunit 2
LB	: Luria Bertanie
MDR3	: Multi Drug Resistance 3
mRNA	: messenger Ribo Nucleic Acid
NCBI	: National Center for Biotechnology Information
NSF	: N-ethylmaleimide-Sensitive Factor
OD	: Optical Density
PDB	: Protein Data Bank
RBL	: Rat Basophilic Leukaemia
RT	: Room Temperaturre

SDS : Sodium Dodecyl Sulphate

SIB : Swiss Institute of Bioinformatics

SNAP : Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein

SNAP-23 : Synaptosome-Associated Protein-23

SNAP-25 : Synaptosome-Associated Protein-25

SNAP-29 : Synaptosome-Associated Protein-29

SNAP-47 : Synaptosome-Associated Protein-47

SNARE : Soluble N-ethylmaleimide-Sensitive Factor Accessory Protein Receptor

SOC : Super Optimal Broth with Catabolite

TBE : Tris Borate EDTA

TGN : Trans Golgi Network

TMH : Transmembrane Helix

VAMP : Vesicle Associated Membrane Protein

VTC : Vesicular Tubular Cluster

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF TABLES

ABSTRACT

Two trans-membrane human proteins namely MDR3 and CLP24 were selected by using online protein databases; UniProtKB, Interpro and NCBI. These proteins have multi-spanning membrane topology that belonged to non-raft regions of the membranes. Membrane topology was predicted using an online tool TMMOD. Similarly, online tools; CSS-Palm, NMT-MYR, GPI-SOM were used to predict the post translational modifications like Palmitoylation, myristoylation and GPI-anchor signals respectively in the various polypeptide segments of the selected proteins. These proteins were then narrowed down to specific polypeptide segments that did not show post-translational modifications. The selected polypeptide sequences are MDR3 (AA, 1-145), MDR3 (AA, 41-145) and CLP-24 (AA, 134-195). Gene expression profile of these proteins were studied in various human normal and cancer cell lines using 'GeneNote' and found that MDR3 and CLP4 were expressed in higher level in liver and lung cells respectively. In-silico fusion proteins hSNAP-23cys^{-}-MDR3, 1-145; hSNAP-23cys^{-}-MDR3, 41-145 and hSNAP-23cys^{-}-CLP24, 134-195 were generated using restriction mapper tool and then the fusion proteins were checked for membrane topology and absence of any post-translational modifications in them.

RNA was isolated from HeLa and A549 cells using Trizol reagent and then its quality was checked using Nanodrop. The Amount of RNA recovered was 8.13 ug/million HeLa cells and 5.76 ug/million A549 cells. These RNA were reverse transcribed into cDNA and then complementary DNA was tested by agarose gel electrophoresis and performing PCR for human GAPDH gene. Specific primers and QIAGEN Onestep PCR kit were used to isolate the DNA fragments for MDR3 (AA, 1-145) and CLP24 (AA, 134-195). These DNA amplicon could be further used to generate fusion protein of SNAP-23 whose expression could then be studied in specific host cell lines after successful transfection. Though the study of expression of the fusion protein remains to be tested in cell lines, bioinformatics studies showed that these fusion proteins would not be signaled to raft region of membrane and hence may prevent the exocytosis process mediated by SNAP-23 proteins in raft regions. This study has helped in exploring the role of cysteine molecules and their palmitoylation in targeting and anchoring of t-SNARE SNAP-23 molecules to the lipid raft region of the membrane by bioinformatic approach where membrane fusion is supposed to occur by SNARE complex.

Key Words: MDR3, CLP24, UniProt, InterPro, NCBI, TMMOD, GeneNote, SNAP-23, SNARE

1. INTRODUCTION

1.1 Background

The array of proteins that any cell will produce (i.e., its "proteome") is, ultimately, a function of its regulated genetic constitution. Once a cell is developmentally committed, however, the correct routing of the proteins that it expresses is just as important to cell function and viability as is the genetic machinery that supports protein expression. Transcription factors, for example, must be routed into the nucleus; membrane proteins must associate correctly with the appropriate organelle; and each cytoplasmic compartment must acquire the proteins and other constituents that render its function.

Protein trafficking is not a unidirectional process. All proteins that are destined for export from the cytosol contain, however transiently, specific peptide sequences that signal them for translocation across or, in the case of membrane proteins, into the cell or organellar membrane. The trafficking of proteins, lipids, and other materials among various cell compartments is quick and energetic. Much of this traffic is mediated by vesicles or other membrane carriers that bud from one compartment and fuse with another (Bonifacino & Glick, 2004).

Membrane fusion, one of the most fundamental processes in life, occurs when two separate lipid membranes merge into a single continuous bilayer. Central to the process of membrane fusion are SNARE proteins, which form a complex known as the core or SNARE complex and are localized to various intracellular organelles and membranes (Sollner *et al*., 1993). Specific SNAREs present on two opposing membranes interact to form a highly stable ‘SNARE complex’; the formation of this protein complex is tightly coupled to membrane fusion. 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 (Hanson *et al*., 1997; Sutton *et al*., 1998). SNARE proteins are classified into two classes based on its membrane localization. They are v-SNARE (vesicle membrane associated) and t-SNARE (target membrane associated).

In cells of the immune system, vesicle trafficking, and thereby SNARE function is important for various cellular responses. Some of the responses include delivery of receptors to and from the cell surface, constitutive secretion of immune mediators, phagocytosis of particulate bacteria, endocytosis of membrane associated cargo, release of stored inflammatory mediators and pre-made products from secretory granules. Many cells of the immune system are specialized secretory cells, whose function depends on regulated exocytosis. Exocytosis is mediated by vesicular transport involving

the sorting of specialized cargo into the secretory granules (SGs), thereby generating the transport vesicles; their transport along the microtubules and eventually their signal-dependent fusion with the plasma membrane.

In most SNAREs, SNARE motifs are located adjacent to a transmembrane domain at the C terminal end. Exceptions include SNAREs that lack a transmembrane domain and contain two SNARE motifs, instead of one, which are connected by a flexible linker, such as mammalian SNAP-25, SNAP-23 and SNAP-29 (Hess *et al*., 1992; Ravichandran *et al*., 1996). SNAP-23 is a t-SNARE, expressed in all non-neuronal cells and has been implicated in regulated exocytosis in cell types such as adipocites and mast cells (Rea *et al*., 1998; Vaidyanathan *et al*., 2001). It is a peripheral membrane protein which lacks a transmembrane domain but contains two SNARE motifs (coiled-coiled domains) linked by a cysteine rich region. It is a component of the cellular mechanism required for specific membrane fusion and targeting of intracellular vesicles and interacts with the other SNARE proteins. In addition to, or as a replacement for, hydrophobic protein domains, membrane association can be mediated by lipidic anchors, which can either be permanent co-translational additions or post-translational modifications under dynamic enzymatic control. These lipid modifications include (1) glycophosphatidylinositol (GPI) anchors; (2) N-terminal myristic acid tails; (3) cysteine acylation; (4) isoprenylation; and (5) the addition of C-terminal sterol moieties.

SNAP-23 has been shown to be present in many immune cells. It is expressed in all non-neuronal cells and has been implicated in regulated exocytosis in cell types such as adipocytes and mast cells (Rea *et al*., 1998; Vaidyanathan *et al*., 2001). SNAP-23 is involved in various protein trafficking events such as GLUT4 trafficking in adipose cells (Rea *et al*., 1998; Kawanishi *et al*., 2000), compound exocytosis in mast cells (Guo *et al*., 1998), polarized protein traffic (Low *et al*., 1998), platelet dense core granule release (Chen *et al*., 2000).

1.2 Current Studies

Current studies on SNAP proteins includes: Association of SNAP-23 to a greater extent with cholesterol-rich region of membranes called rafts in mast cells (Salaun *et al*., 2005). Palmitoylation of five cysteines molecules in the linker region of SNAP-23 next to the sites of induced phosphorylation (Hepp *et al*., 2005). Palmitoylation of these cysteines is thought to anchor SNAP-23 to membrane. But the actual role of these cysteines in targeting of SNAP-23 to membranes or membrane microdomains has not been investigated before.

1.3 Objectives

1.3.1 General Objective

- To study the role of palmitoylation of SNAP-23 in membrane raft association and regulated exocytosis by generating fusion proteins (in-silico) of t-SNARE SNAP-23 using a transmembrane polypeptide domain.

1.3.2 Specific Objectives

- To identify suitable transmembrane domains from non-raft associated proteins from human that do not shows any post-translational modifications.
- To generate fusion protein in-silico and isolation of DNA fragments for the selected polypeptide domains that could be used for generating fusion protein in-vitro.
- To investigate the role of cysteine rich linker region of SNAP-23 in membrane raft association by post-translational modification like palmitoylation.
- To identify the role of SNAP-23 in raft associated membrane fusion event and regulated exocytosis in immune cells like mast cells.
- To study the role of post-translational modifications of proteins in membrane association and signaling.

1.4 Rationale and Scope

Bioinformatics approach will be used as pioneering work in assisting this whole study which saves time and labor with more precision in lab works. The fusion protein that will be created in-silico is believed to be generated in the laboratory and then their expression could be studied in an appropriate host system. Bioinformatic approach is nowadays gaining more popularity in searching database and finding out suitable candidate before applying it to the in-vitro tests. Once the role of palmitoylation of cysteine molecules in the linker region of SNAP-23 and its association with lipid-raft region will be clear, we could answer the mechanism of regulated exocytosis mediated my SNAP-23 proteins in non-neuronal cells. Moreover, the result is also expected to solve the problem type-I anaphylactic shock mediated by uncontrolled degranulation of mast cells. So, if the mechanism of regulated exocytosis in these cells is well understood then it could help in discovering novel ideas in controlling these events by pharmacological approach. One of the reasons for carrying this study is also that we are focused at solving the unanswered question about the possible mechanism of lipid-raft localized exocytosis events expected to be controlled by SNAP-23 and related SNARE proteins.

2 LITERATURE REVIEW

2.1 Membrane Fusion and Vesicular Trafficking

Membrane fusion, one of the most fundamental processes in life, occurs when two separate lipid membranes merge into a single continuous bilayer. Fusion reactions share common features, but are catalyzed by diverse proteins (Jahn *et al*., 2003). Lipid bilayers fusion in an aqueous environment is a two-step process. First, the membranes are brought into close proximity where counteracting electrostatic forces need to be overcome before the lipids of the proximal leaflets can interact. Second, the boundary between the hydrophilic and hydrophobic portion of the bilayer is destabilized. Non-bilayer transition states are generated that culminate in the formation of an aqueous fusion pore (Kozlov and Markin, 1983; Chernomordik *et al*., 1987).

Membrane fusion can be broadly distinguished into three types: (1) Extra- and intracellular fusion of pathogens with host cells (2) Extracellular fusion of eukaryotic cells (3) Intracellular fusion of organelles. These reactions are mediated by dynamic supramolecular assemblies involving conserved protein families (Jahn *et al*., 2003). Intracellular fusion of organelles includes various cellular processes including vesicular traffic along the secretory pathway, organelle inheritance and neurotransmitter release.

Central to the process of membrane fusion are SNARE proteins, which form a complex known as the core or SNARE complex and are localized to various intracellular organelles and membranes (Sollner *et al*., 1993). Specific SNAREs present on two opposing membranes interact to form a highly stable 'SNARE complex'; the formation of this protein complex is tightly coupled to membrane fusion (Sollner *et al*., 1993; Weber *et al*., 1998). 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 (Hanson *et al*., 1997; Sutton *et al*., 1998).

In 1984, two soluble proteins were purified that efficiently mediated protein movement along the vesicle trafficking pathway, N-ethylmaleimide sensitive factor (NSF) and an adaptor protein called Soluble NSF attachment protein (SNAP) (Balch *et al*., 1984; Block *et al*., 1988). These proteins were found to act in many intracellular pathways except that of fusion events with mitochondria and peroxisomes which appear to utilize a set of unrelated proteins. The discoveries of the SNARE proteins lead to the proposal of a SNARE hypothesis. This hypothesis dictates that a unique pairing of cognate vesicle (v-) and target (t-) SNAREs ensures that specific transport vesicles dock and fuse with their

appropriate target membranes with the subsequent dissociation of the SNARE complex by the ATPase activity of NSF driving membrane fusion (Sollner *et al*., 1993).

Eukaryotic cells contain a profusion of intracellular compartments bounded by membranes (Figure 1). The trafficking of proteins, lipids, and other materials among these compartments is quick and energetic. Much of this traffic is mediated by vesicles or other membrane carriers that bud from one compartment and fuse with another (Bonifacino & Glick, 2004). Cargo-laden vesicles are created when they are sculpted out of the membrane of one compartment by the assembly of coat proteins. To deliver their cargoes, the vesicles must traffic to their destinations, lose their coats, and fuse with the target compartment's membrane. Carrying out these processes across the many trafficking pathways within a typical eukaryotic cell requires more than a hundred proteins that can, collectively, be considered to represent the trafficking machinery. For example, coat proteins such as clathrin and COPII have been characterized both functionally and structurally, and the pathways in which they act have been elucidated. Similarly, the membrane anchored soluble NSF (N-ethylmaleimide sensitive factor) acceptor protein receptor (SNARE) proteins required for the merger of vesicle and target membranes have been intensively studied and are known to function through the formation of trans complexes that bring the two membranes into direct apposition.

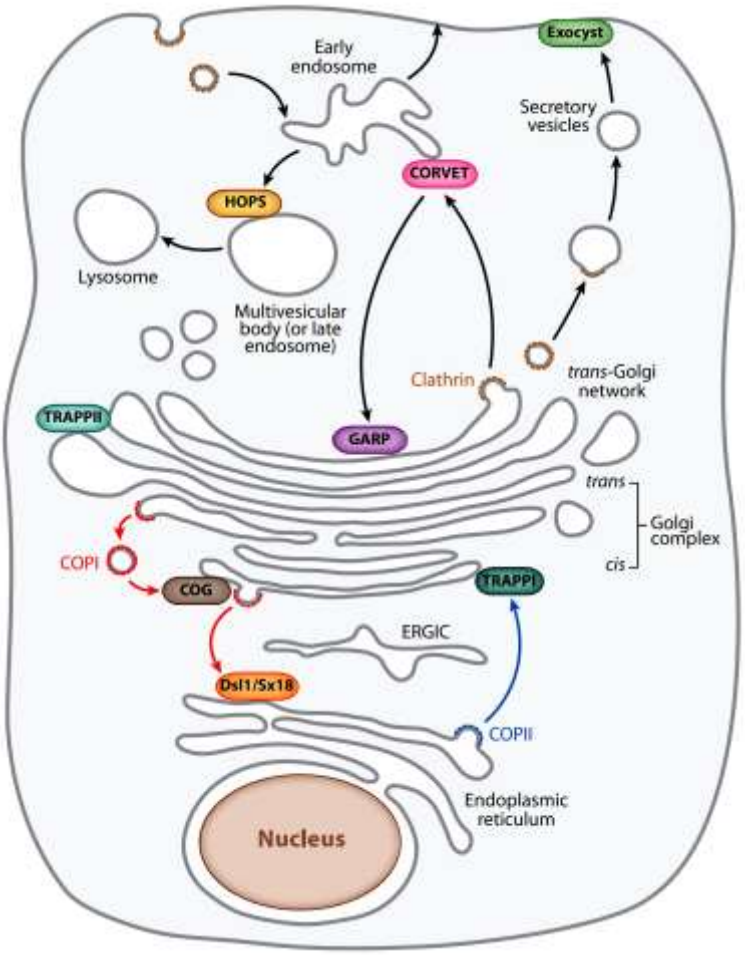


Figure 1:

Trafficking pathways in eukaryotic cells. Some of the coat proteins (COPI, COPII, clathrin) that mediate transport vesicle formation as well as the eight multi-subunit tethering complexes (colored ovals). Abbreviations: COG, conserved oligomeric Golgi complex; CORVET, class C core vacuole/endosome tethering complex; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; GARP, Golgi-associated retrograde protein complex; HOPS, homotypic fusion and vacuole protein sorting (or class C vacuolar protein sorting) complex; TRAPP, transport protein particle (Ref: Bonifacino & Glick, 2004).

2.2 Classification of SNAREs

Due to the localization of these proteins to synaptic vesicles or presynaptic membranes, it was suggested initially that two classes of SNARE proteins existed and were then classified as t- (target) or v- (vesicle) SNAREs depending on the membrane which they are located at (Sollner *et al*., 1993).

I. v- SNAREs (Vesicle associated): These are incorporated into the membranes of transport vesicles during budding. These are generally small, type II integral membrane proteins that make up the vesicle associated membrane protein (VAMP)/synaptobrevin family.

II. t- SNAREs (Target associated): These are located in the membranes of target compartments. These consist of members of the Syntaxin family & the SNAP-25 family.

When describing homotypic fusion, however, these descriptions are ambiguous since t-SNAREs can be localized to vesicles and v-SNAREs to target membranes. Accordingly, in light of the elucidation of the crystal structure of the SNARE complex, SNAREs have recently been categorized more accurately as either R-SNAREs (containing an arginine at the ionic layer position) or Q-SNAREs (containing a glutamine at the ionic layer position) depending on whether a highly conserved central residue of the SNARE motif is either an Arginine (R) or Glutamine (Q) residue. In general, most v-SNAREs contain a conserved arginine residue and thus are R-SNAREs, while most t-SNAREs (e.g., Syntaxins, SNAP-25 isoforms) contain glutamine/aspartate residues and are thus Q-SNAREs (Weimbs T, 1997; Fasshauer *et al*., 1998). It has now been shown that three Q-SNAREs and one R-SNAREs are required for fusion events (McNew *et al*., 2000).

2.3 Structure of SNARE Proteins

SNARE proteins comprise a superfamily of mostly membrane bound proteins with at least 24 members in yeast and 35 members in mammals (Bock *et al*., 2001; Hong W, 2005). The characteristic feature of all SNAREs is the presence of a stretch of ~60 amino acids arranged in heptad repeats that are referred to as a SNARE motif (Hardwick and Pelham, 1992). In most SNAREs, SNARE motifs are positioned adjacent to a transmembrane domain at the C terminus. Exceptions include SNAREs that lack a transmembrane domain and contain two SNARE motifs, instead of one, which are connected by a flexible linker, such as mammalian SNAP-25, SNAP-23 and SNAP-29 (Hess *et al*., 1992; Ravichandran *et al*., 1996). Most SNAREs are small type II membrane proteins that have typically 20 to 30% protein similarity as a superfamily. Sequence

alignments of the most conserved regions in the core domains of the neuronal SNAREs were mapped onto the crystal structure of the SNARE complex. Mutations in these layers are known to reduce complex stability and can cause defects in membrane trafficking, even in distantly related SNAREs (Fasshauer *et al*., 1998b). This allows the coiled-coil sequences of SNAREs to form an α-helix bundle. The interacting amino acid residues at the core hydrophilic ionic layer are flanked by hydrophobic leucine-zipper layers (Sutton *et al*., 1998). It is thought that clamping of the membranes and initiation of the fusion of the membrane proceeds from the N-terminal to the C-terminal end (Hanson *et al*., 1997). The majority of the protein is exposed in the cytoplasm, followed by a single membrane-spanning hydrophobic region and a few amino acids facing either the lumen of an intracellular compartment or the extracellular side. Some SNAREs (SNAP-23, SNAP-25, SNAP-29, Syn11 and Ykt6) do not have a C-terminal membrane spanning domain, but are instead attached to the membrane by prenylation in Ykt6 (Fukasawa *et al*., 2004), by palmitoylation of cysteine residues in SNAP-25, Ykt6 and Syn11 (Fukasawa *et al*., 2004; Veit *et al*., 1996; Prekeris *et al*., 2000) and/or by interaction with other SNAREs that are anchored by C-terminal tails (Vogel *et al*., 2000). Individual SNARE proteins are unfolded, but they spontaneously assemble into a remarkably stable four-helix bundle that forms between membranes as a "trans-SNARE complex" which is also known as a "SNAREpin" (Antonin *et al*., 2002). The assembly forces membranes closely together as the complex zippers up and drives membrane fusion. The SNARE complex is a stable structure having a melting temperature greater than 90^{o}C and is resistant to denaturation by SDS and botulinum neurotoxins (Hayashi *et al*., 1994; Poirier *et al*., 1998).

A recent study demonstrates that generalized disruption of SNARE machinery inhibits retroviral particle production, suggesting that there is an important role for the SNARE proteins in HIV assembly and release (Joshi *et al*., 2011).

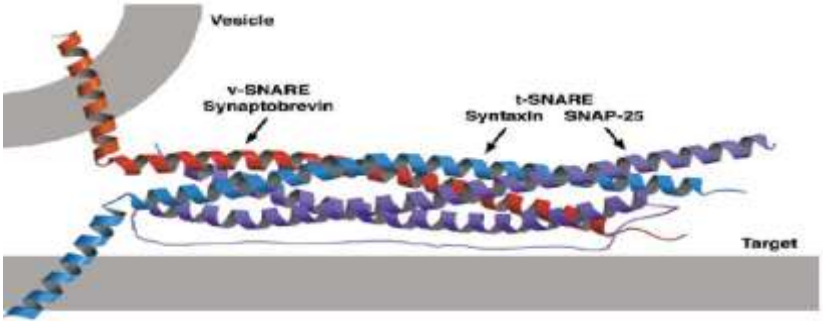


Figure 2: Crystal structure of synaptic trans-SNARE complex. The structures of the two membrane anchors and of the peptide that links the two SNAP-25 α-helices are hypothetical (Ref: Sutton *et al*., 1998)

2.4 Known SNARE Complexes (SNAREpins) in Mammalian Cells

Several SNARE complexes have been defined to function in various transport events in the secretory and or endocytic pathways of mammalian cells (Fig. 3). The complex consisting of Syn5 (Qa), GS27 (Qb), Bet1 (Qc), and Sec22b (R) appears to function in mediating homotypic fusion of ER-derived COPII vesicles into larger transport intermediates referred to as EGTC (ER-Golgi transport container), ERGIC (ER-Golgi intermediate compartment), or vesicular tubular cluster (Zang *et al*., 1997; Zang *et al*., 1999). EGTCs are dynamic structures that undergo maturation events (including recycling of proteins back to the ER) as they move towards the Golgi apparatus (Horstmann *et al*., 2002) receiving the recycling traffic (Hirose *et al*., 2004). The endosomal compartments are known to be integrated with the secretory pathway by retrograde traffic from various endosomal compartments to the TGN. The major SNARE complex functioning in the retrograde traffic from early /recycling endosomes to the TGN consists of Syn16 (Qa), Vti1a (Qb), Syn6 (Qc), and VAMP-4 (R). The same TGN t-SNARE interacts also with VAMP-3, which plays a minor role in this retrograde recycling pathway (Mallard *et al*., 2002). Several SNARE complexes are implicated in the endocytic pathway. Syn13 is likely the major Qa SNARE functioning in the early/sorting endosome and it interacts with SNAP-25 (Qb and Qc) and VAMP-2 (R) to regulate fusion of early/sorting endosomes (McBride *et al*., 1999, Sun *et al*., 2003). VAMP-2 is the v-SNARE in this fusion event. Exocytic traffic from the TGN to the cell surface is not only important for constitutive secretion and biogenesis of the plasmamembrane but also for regulated traffic in diverse physiological processes. The neuronal SNARE complex consisting of Syn1, SNAP-25, and VAMP-2 is the best studied and has served as a paradigm for other vesicular transport events (Sollner *et al*., 1993; Sutton *et al*., 1998; Jahn *et al*., 2003; Sudhof TC, 2004). The functionality of VAMP-2 as the v-SNARE, the assembly of the t-SNARE from Syn1 and SNAP-25, as well as the activity of the assembled trans-SNARE complex in mediating synaptic vesicle fusion with the plasma membrane are subjected to diverse regulatory mechanisms. This SNARE complex is also the converging point for many cellular regulations on presynaptic events in the brain. A major mode of insulin action is to mobilize glucose transporter 4 (GLUT4) by stimulating fusion of GLUT4 containing intracellular vesicles with the plasmamembrane. In this case, VAMP-2 functions as a v-SNARE for GLUT4-containing vesicles and it interacts with t-SNARE assembled from Syn4 and SNAP-23 (Bryant *et al*., 2002). VAMP-8 was recently shown to be the premier v-SNARE of zymogen granules in pancreatic acinar cells and it mediates regulated fusion with the apical surface, interacting with t-SNARE assembled from Syn4 and SNAP-23 (Wang *et al*., 2004). The Syn4/SNAP-23 t-SNARE may also

mediate the fusion of secretory lysosomes with the plasmamembrane by interacting with VAMP-7 as the v-SNARE (Rao *et al*., 2004).

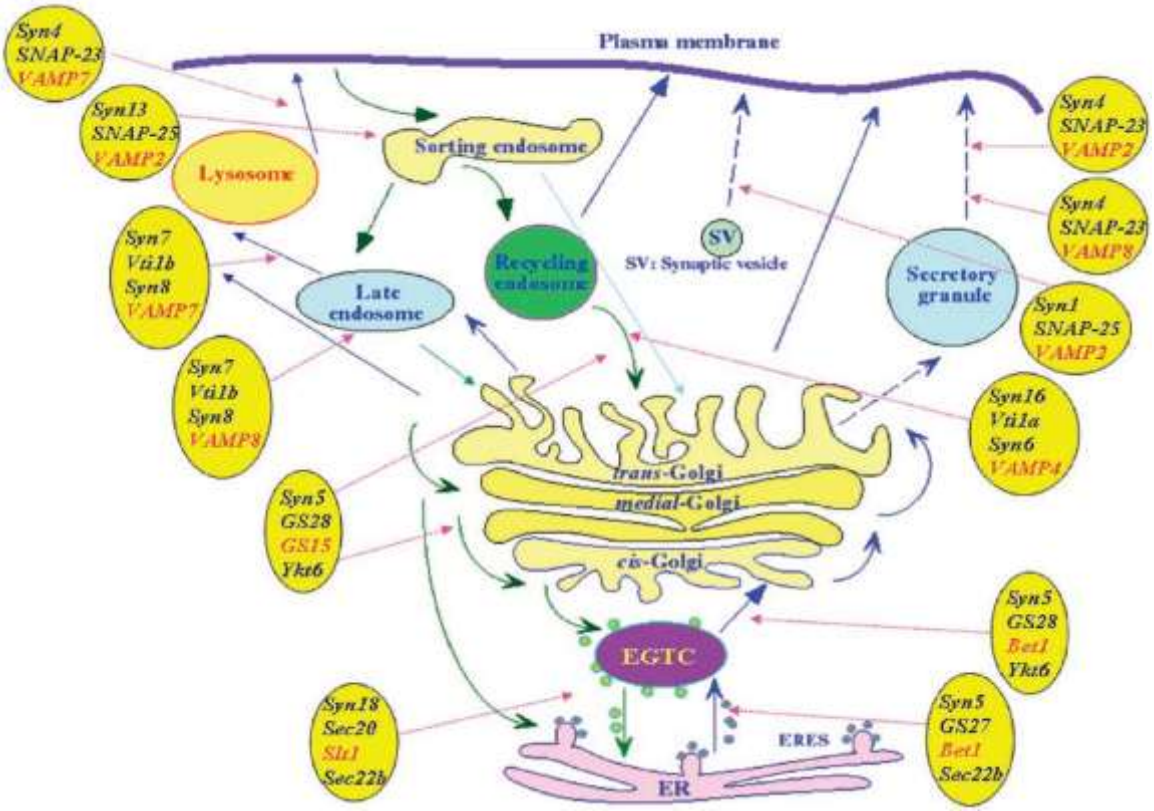


Figure 3: Schematic summary of known mammalian SNARE complexes and their site of action in exocytic and/or endocytic pathways. Potential v-SNAREs are shown in red (Hong W, 2005).

2.5 Syntaxin Family

One of the first SNARE elements to be identified, syntaxin 1, was found in the plasma membrane of neuronal cells (Bennett *et al*., 1993). Since then, at least 20 syntaxin isoforms have been described across the animal and plant kingdoms. Typically, syntaxins have molecular weights of approximately 35 kDa and fall into Q- or t-SNARE classification. Syntaxins are type II integral membrane proteins containing a single C-terminal transmembrane domain, a SNARE domain (referred to as H3) and a N-terminal regulatory domain, Habc (Hirai *et al*., 1993). The H3 domain is highly conserved among all syntaxin isoforms and is required for binding SNAP-25 and VAMP (Calakos *et al*., 1994). Syntaxin, particularly syntaxin 1, is believed to be regulated by the SNARE-associated protein, Munc18. Munc18 binds to syntaxin 1 and holds the protein in a closed conformation by collapsing the H3 helix (Hata *et al*., 1993). The dissociation of

syntaxin from Munc18 allows for the conformation of syntaxin opening and interacting with other SNARE proteins (eg., VAMP-2 and SNAP-25). The distribution of syntaxin varies upon isoforms, for example, syntaxin 1A is primary localized to the neuronal plasma membrane and is concentrated in synapses (Bennett *et al*., 1993). Syntaxin 2, 3 and 4 have also been shown to be localized at the plasma membranes of cells, however, they have a much wider tissue distribution (Bennett *et al*., 1993). Syntaxin 5 and 6 have been found to be localized to the Golgi membrane as well as endosomal structures (Banfield *et al*., 1994). Moreover, syntaxin 7 is involved in the fusion of late endosomes and lysosomes. In endosomal membranes, it has been shown to form complexes with VAMP-8 and syntaxin 8 (Advani *et al*., 1998).

The mechanism of insertion of tail-anchored proteins into the ER membrane can occur by a number of mechanisms that differ in ATP dependence and receptors (Borgese *et al*., 2003). However, the exact requirements for syntaxin insertion into the ER membrane have not been analyzed. The C-terminal membrane anchor of syntaxins 1–4 is preceded by a membrane proximal ~60-amino acid coiled-coil region that participates in SNARE complex assembly. The function of specific syntaxin proteins is required for defined exocytosis pathways. For example, syntaxin 1 functions in exocytosis pathways such as presynaptic neurotransmitter release (Blasi *et al*., 1993) , 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 (Volchuk *et al*., 1996)

2.6 Synaptobrevin Family

The synaptobrevin family, also known as vesicle associated membrane proteins (VAMPs) are small integral membrane proteins of secretory granules with molecular weights on average of 11-13 KDa (Baumert *et al*., 1989). They are classified as members of v- or R-SNAREs. They have two domains, an N-terminal region exposed to the cytoplasm and a C-terminal transmembrane domain including a conserved 60 amino acid central sequence that enables binding to syntaxin and SNAP-25. Two isoforms have been identified in the central nervous system, VAMP-1 and VAMP-2. They are distributed within different regions of the brain and are highly homologous with approximately 80% amino acid identity (Sudhof TC, 1995). These proteins have been shown to be the major constituents of synaptic vesicles and other secretory granules as well as an essential component of the exocytotic fusion machinery. VAMP-3, also known as cellubrevin, is a non-neuronal isoform that has been identified and localized to the endosomal membrane pool and ubiquitously expressed. Interestingly, VAMP-5 has been reported to

be expressed in skeletal muscle and heart and localized to the plasma membrane in these tissues (Zeng *et al*., 1998).

VAMP-7 and VAMP-8 have been shown to be associated with endocytic or intracellular vesicle-trafficking processes (Advani *et al*., 1999; Antonin *et al*., 2000); however, recent reports have also delivered evidence for exocytotic functions (Paumet *et al*., 2000; Logan *et al*., 2006). VAMP-7 is the most crucial v-SNARE for the release of mediators from secretory lysosomal compartments in two human inflammatory cell types, eosinophils and neutrophils (Logan *et al*., 2006). VAMP-7 is found to be essential for NK cell-mediated target cell killing mechanism. VAMP-7 also plays a major role in docking and fusion of secretory lysosomes to the plasma membrane in hematopoietic cells (Marcet-Palacios *et al*., 2008)

VAMP-8/endobrevin is the smallest v-SNARE, consisting of 100 amino acids and its sequence is 32% identical to that of VAMP-2/synaptobrevin (Advani *et al*., 1998). VAMP-8 was originally identified as a v-SNARE in the pathway of endocytosis by immunocytochemical and electron microscopic localization. VAMP-8 makes a SNARE complex with 3 t-SNAREs {Syntaxin7 (Qa), Vti 1b (Qb), and Syntaxin8 (Qc) } that reside on endosomal membranes, and hence was strongly suggested to be involved in homotypic membrane fusion between early endosomes and between late endosomes (Antonin *et al*., 2000; Fasshauer *et al*., 1999). VAMP-8 is a major v-SNARE for exocrine secretion (Cosen-Binker *et al*., 2008; Wang *et al*., 2004). In addition to this VAMP-8 plays a role in regulated exocytosis from platelets and mast cells (Lippert *et al*., 2007; Ren *et al*., 2007; Puri and Roche, 2008).

2.7 SNAP-23/25 Family

Joining syntaxin and VAMP as a key component of the minimum machinery required for exocytosis is the synaptosome-associated protein of 25,000 daltons (SNAP-25). SNAP-25 is a membrane bound protein anchored to the cytosolic face of membranes by palmitoyl side chains (Lin and Scheller, 2000). SNAP-25 is classified as a Q-SNARE contributing two α-helices to the formation of the fusion complex. In neurons, SNAP-25 assembles with syntaxin 1 and VAMP-2. A ubiquitously expressed isoforms and functional homologue of SNAP-25 is the synaptosome-associated protein of 23,000 daltons, SNAP-23. In addition to SNAP-23 and SNAP-25, there are two other members of the family. SNAP-29 and SNAP-47, expressed in a variety of tissues, lack a membrane anchor and may be localized to a vesicle pool which is not confined to a specific organelle (Wong *et al*., 1999; Holt *et al*., 2006).

2.7.1 SNAP-23

Using a yeast two-hybrid screen, SNAP-23 was first identified in human B lymphocytes (Ravichandran *et al*., 1996) and later found to be ubiquitously expressed in many tissues (Araki *et al*., 1997; Mollinedo and Lazo, 1997). SNAP-23 is a t-SNARE, expressed in all non-neuronal cells and has been implicated in regulated exocytosis in cell types such as adipocytes and mast cells (Rea *et al*., 1998; Vaidyanathan *et al*., 2001), and may also have a general function in constitutive exocytosis (Leung *et al*., 1998). For example, SNAP-23 is involved in diverse protein trafficking events such as GLUT4 trafficking in adipose cells (Rea *et al*., 1998; Kawanishi *et al*., 2000), compound exocytosis in mast cells (Guo *et al*., 1998), polarized protein traffic (Low *et al*., 1998), platelet dense core granule release (Chen *et al*., 2000). It is a peripheral membrane protein which lacks a transmembrane domain but contains two SNARE motifs (coiled-coiled domains) linked by a cysteine rich region. It is a component of the cellular mechanism required for specific membrane fusion and targeting of intracellular vesicles and interacts with the other SNARE proteins (Cabaniols *et al*., 1999). SNAP-23 associates to a greater extent with cholesterol-rich membranes in mast cells. SNAP-23 has five cysteines in its linker region next to the sites of induced phosphorylation and has been shown to get palmitoylated (Hepp *et al*., 2005) although to a lesser degree than that of SNAP-25 (Vogel *et al*., 1999). Plamitoylation of these cysteines is thought to anchor SNAP-23 to membrane. But the actual role of these cysteines in targeting of SNAP-23 to membranes or membrane microdomains has not been investigated before. This palmitoylation is reversible, since treatment of permeabilized platelets with a depalmitoylation agent (acyl-protein thioesterase 1) can cause SNAP-23 translocation from the membrane to the cytosol (Sim *et al*., 2007). SNAP-23, however, differs from SNAP-25 in that the former is resistant to BoNT/A and /E cleavage (Chen *et al*., 1997; Sadoul *et al*., 1997). Using a yeast two-hybrid system, human/mouse SNAP-23 was shown to interact with Syntaxin-1 and -4 strongly, with Syntaxin-2 moderately, and with Syntaxin-3 weakly (Araki S, 1997).

SNAP-23 has various regions whose specific functions have been identified. The C and N-terminal domains are found to play very important roles in forming binary SNARE complexes with other SNARE proteins like VAMP and Syntaxins (Vaidyanathan *et al*., 2001). The first 18 amino acids of SNAP-23 harbour a major binding site for both syntaxin and VAMP and the presence of the first coiled-coil region alone is insufficient to allow SNAP-23 binding to syntaxin and VAMP. The extreme carboxyl terminus of SNAP-23 is not required for the binding of SNAP-23 to syntaxin 4 but is essential for the binding of SNAP-23 to VAMP-2. In-vivo studies have also shown that the VAMP-binding

domain of SNAP-23 is required for regulated exocytosis from mast cells (Vaidyanathan *et al*., 2001).

Two isoforms of the vesicle-membrane fusion protein SNAP-23 namely SNAP-23A and SNAP-23B have been identified in human neutrophils. SNAP-23A is similar to recently cloned SNAP-23, and a novel isoform, termed SNAP-23B. SNAP-23B results from a deletion of 159 bp of the SNAP-23A cDNA, encoding a protein of approximately 17.8 KDa. SNAP-23B is identical to SNAP-23A, but lacks 53 amino acid residues, from 90 to 142 which is a target sequence in SNAP-23A for post-translational fatty acid acylation, suggesting that the two isoforms can differ in their capacity to interact with membranes. SNAP-23A proteins has two potential target threonine residues at 144 and 188 position which gets phosphorylated by cAMP/cGMP-dependent protein kinase whereas SNAP-23B protein has only one threonine residue at position 135 that gets phosphorylated (Mollinedo *et al*., 1997).

Figure 4: Cartoon structure of SNAP-23, highlighting the location of phosphorylation sites (Ser95 and Ser120) in relation to the cysteine-rich linker region of SNAP-23 and the amino and carboxyl-terminal coiled-coil domains (Hepp *et al*., 2005).

2.7.2 SNAP-25

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. As shown in figure 2, SNAP-25 has a cluster of cysteine residues at the N-terminus of the linker domain (residues 85-120) which are palmitoylated (Hess *et al*., 1992; Koticha *et al*., 1999). Some research suggested that it is through these hydrophobic palmitate molecules that SNAP-25 attaches to the presynaptic membrane (Gonzalo *et al*., 1998; Koticha *et al*., 1999). However, the question of when and where SNAP-25 is palmitoylated is still unclear; experimental results have been inconsistent or even contradictory. Some experiments showed that palmitoylation of SNAP-25 required a functional secretory pathway, which suggested that SNAP-25 was transported through the ER and the Golgi apparatus to the

presynaptic membrane and that palmitoylation occurred during transit (Gonzalo *et al*., 1998). However, other experiments showed that palmitoylation defective forms of SNAP-25 could attach to a membrane when syntaxin 1A was present (Vogel *et al*., 2000; Washbourne *et al*., 2001; Sorensen J, 2005). In these cases, it was suggested that SNAP-25 and syntaxin could form a precomplex through which SNAP-25 attached to membrane. Moreover, it was found that SNAP-25 was palmitoylated by a membrane bound palmitoyltransferase (Huang *et al*., 1999). These observations suggest that SNAP-25 is palmitoylated after its syntaxin mediated membrane association, rather than right after its synthesis as other experiments suggested. Furthermore, they indicate that SNAP-25 can associate to a membrane indirectly through other proteins, independent of palmitoylation.

The first identified SNAP-25 proteins were termed SNAP-25A and SNAP-25B; these isoforms are highly homologous, and generated by alternative splicing, and differ by only 9 amino acids located in the central part of the molecule. This SNAP-25 domain encodes the portion of the protein which is a target for palmitoylation (Bark IC, 1993; Bark *et al*., 1994; Veit *et al*., 1996). Both SNAP-25A and SNAP-25B retain the four cysteine residues, thought to be essential for palmitoylation, but in a different spatial organization and sequence context, which may affect the dynamics of fatty acid acylation, and consequently the interactions of SNAP-25 with membranes (Bark IC, 1993). Interestingly, it has been reported recently that SNAP-25A expressed in *Escherichia coli* is also an iron-sulfur protein that binds to an iron-sulfur cluster using the cysteine residues. Therefore, SNAP-25A uses the same cysteine residues to bind two different prosthetic groups (i.e., iron-sulfur cluster and palmitate) (Huang *et al*., 2008). It has also been suggested that, since the binding sites of these two prosthetic groups overlap, these two modifications occur at different times and probably at different sites in the cell (Huang *et al*., 2008).

SNAP-25A/B exhibits a restricted expression pattern, being most abundant in neuronal and neuroendocrine cells, and these proteins have a specialized function in fast regulated exocytosis pathways, such as synaptic vesicle exocytosis (Schiavo *et al*., 1993). Furthermore, syntaxin has been suggested to be required for efficient trafficking of SNAP-25 to the plasma membrane.

SNAP-25A	80 DLGK**C**C**C**GLFI**C**P**C**NKLKSSD 99
SNAP-25B	80 DLGKF**C**GL**C**V**C**P**C**NKLKSSD 99
SNAP-23	75 ELNK**C**C**C**GL**C**V**C**P**C**NRTKNFE 94

Figure 5: Comparison of the cysteine cluster of SNAP-25A, SNAP-25B and SNAP-23 (Huang *et al*., 2008)

2.7.3 SNAP-29

SNAP-29, another SNAP-25 related isoform, is found in a wide variety of tissues, including pancreas. Because of its intracellular membrane association in non-neuronal cells and its interactions with most members of syntaxin family, SNAP-29 has been considered to be a ubiquitous cytoplasmic SNARE protein involved in general membrane trafficking steps. However, its exact function in SNARE-mediated membrane fusion remains elusive (Su *et al*., 2001).

SNAP-29 is localized to multiple membranes (rather than anchoring to one specific compartment), mainly those of intracellular structures such as the Golgi, trans-Golgi network, and endosomal compartment. It is also found on the plasma membrane (Steegmaier *et al*., 1998). Its structure is somewhat atypical for a SNAP-25 isoform. It lacks palmitoylation sites. Additionally, since SNAP-29 in vitro binds many different Syntaxin isoforms, it has been proposed that it mediates numerous intracellular fusion events with multiple Syntaxins and VAMPs which are localized to distinct membranes (Steegmaier *et al*., 1998). SNAP-29 also contains two peptide motifs found in proteins involved in endocytic vesicle trafficking and clathrin coat formation which are absent in SNAP-23 and SNAP-25 (Rotem-Yehudar *et al*., 2001). It has also been reported that SNAP-29 is present at synapses, interacts directly with syntaxin-1A, competes with α-SNAP for binding to the SNARE complex, and consequently modulates synaptic transmission by inhibiting disassembly of the SNARE complex (Su *et al*., 2001).

2.7.4 SNAP-47

Comprehensive proteomic analysis of synaptic vesicle revealed another protein/isoform now termed SNAP-47 consisting of two SNARE motifs belonging to the Qb and Qc subfamilies, respectively. These two motifs are connected by a linker region, thus identifying the protein as a new member of the SNAP-25 subgroups, which had been previously registered in the data base as containing a single SNARE motif and termed SVAP1 (Holt *et al*., 2006). The closest mammalian homologue of this protein appears to be SNAP-29, a SNARE protein that contains two SNARE motif connected by a linker region (shown in figure 6*A*). SNARE motifs in SNAP-47 contain a glutamine (Q) in the position aligning with the highly conserved “0” layer (Holt *et al*., 2006).

Sequence comparison of SNAP-47 with other SNAP-25 homologues shows that SNAP-47 has a longer N-terminal stretch and a markedly extended loop between its two SNARE motifs (shown in figure 6*B*). Similar to other SNAP-25 homologues, a typical transmembrane domain is missing. However, unlike SNAP-25 and SNAP-23, SNAP-47

lacks a conserved stretch of cysteine residues in the linker region connecting the two SNARE motifs. SNAP-47, similar to its closest relative SNAP-29, is not palmitoylated. Furthermore, SNAP-47 is not cleaved by botulinum neurotoxins (BoNT) A or E light chains, two bacterial proteases that are known to cleave SNAP-25 but not SNAP-29 (Schiavo *et al*., 2000). The distribution pattern of SNAP-47 resembles that of synaptobrevin 2 (also referred to as VAMP-2), a known synaptic vesicle-resident R-SNARE that plays a role in neuronal exocytosis (Jahn *et al*., 2003). SNAP-47 has been found to have a widespread distribution that includes synaptic vesicle and also other intracellular membrane pools (Holt *et al*., 2006).

SNAP- 47 can form stable SNARE complexes with neuronal SNAREs in vitro, and catalyzes fusion of proteoliposomes. Unlike SNAP-25, whose expression is limited to neurons and neuroendocrine cells and tissues, SNAP-47 is expressed at higher levels in a wide variety of tissues like brain, liver, kidney, heart, testis and at a lower level in tissues like lungs, skeletal muscle and spleen. SNAP-47 forms a ternary SNARE complex with syntaxin1a and synaptobrevin 2 (Holt *et al*., 2006).

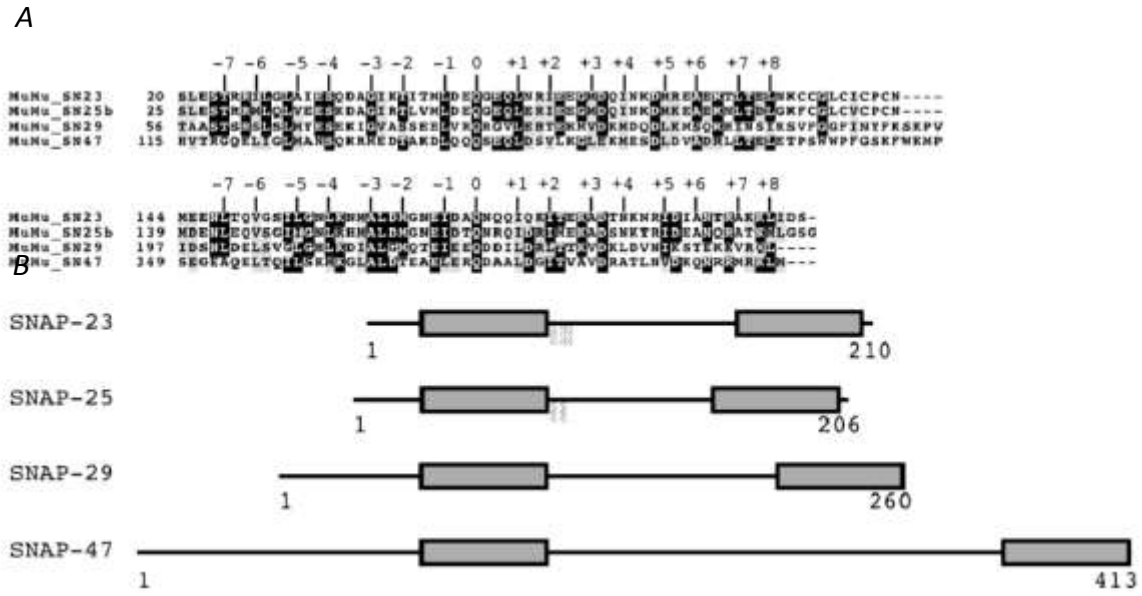


Figure 6 (*A):* Alignment of the two SNARE motifs of the Qbc-SNAREs SNAP-23, SNAP-25b, SNAP-29 and SNAP-47 from mouse. The well conserved heptad repeat layers -7 to +8 are indicated. (*B):* Schematic view of the domain structure of Qbc-SNAREs. All SNAP-25 homologues contain tandem SNARE motifs (boxed). Note the palmitoylation sites in the linker region indicated on SNAP-23 and SNAP-25 (Holt *et al*., 2006)

2.8 SNARE Complex Formation: Vesicle Docking Mediated by Rab Proteins and Fusion by SNAREs

To target vesicle fusion to distinct membrane sub-domains, vesicle tethering is locally restricted. Tethering proteins can directly interact with SNAREs and provide an additional layer of specificity and contribute to high-fidelity fusion. Vesicle tethering precedes SNARE complex formation, and at least some tethering proteins play an active role in SNARE complex formation (Shorter *et al*., 2002). Tethering factors have been identified in nearly all intracellular membrane transport steps and employ Rab proteins and some of their effectors (Grosshans *et al*., 2006). In general, Rabs and their tethering effectors provide the local environment for efficient membrane fusion. In the presence of cognate tethering proteins, Rabs directly link membranes that subsequently undergo homo- or heterotypic fusion (Cai *et al*., 2007). Rabs are small compartment-specific GTPases that continuously cycle between the cytosol (in an inactive GDP-bound state) and membranes (in an active GTP-bound state). Membrane binding of an activated Rab protein at distinct intracellular compartments is followed by the recruitment of effectors and is temporally restricted due to GTP-hydrolysis. Rabs recruit many functional diverse effectors that operate in cargo sorting, vesicle motility, modeling of membrane sub-domains, regulation of SNARE activity or tethering. Hence, in an orchestrated manner with their effectors, Rabs appear to co-ordinate the sequential steps in distinct intracellular trafficking pathways.

Upon SNARE complex assembly, conformational changes take place and α-helical content increases (Fiebig *et al*., 1999; Fasshauer *et al*., 1997a/b). The resulting coiled-coil SNARE complex contains four amphipathic α-helices; one SNARE domain is contributed by the R-SNARE and one each from the Qa, Qb, and Qc SNAREs as shown in the figure 2 (Sutton *et al*., 1998; Dietrich *et al*., 2005). The formation of four helix bundles during complex formation is a defining characteristic conserved among the SNARE protein superfamily (Fasshauer *et al*., 1998). Both synaptic and yeast vacuole SNARE complexes have this four helical domain structure (Sutton *et al*., 1998; Fukuda *et al*., 2000; Dietrich *et al*., 2005). During complex formation, the SNARE proteins are thought to zipper up from the membrane-distal NH_2 termini to the membrane-proximal COOH termini, thus driving fusion of opposing membranes (Hanson *et al*., 1997 a/b; Hay and Scheller, 1997). $Ca2^+$ triggers the full zippering of the coiled-coil complex, which results in membrane fusion and release of vesicle contents.

During fusion, opposing membranes that are in contact proceed through a series of intermediates. As a result, an aqueous fusion pore is formed that connects the distal

leaflets of the fusing membranes while maintaining a lipidic seal between the distal and the proximal leaflets during the reaction. An intuitive and physically well founded model is provided by the stalk hypothesis that, although not without challenges, describes fusion as an ordered sequence of lipidic non-bilayer transition states (as shown in figure 7).

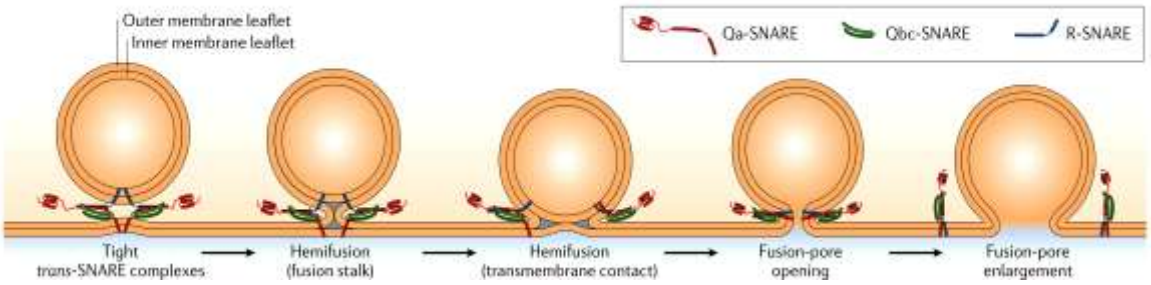


Figure 7: Hypothetical transition states in SNARE-mediated fusion according to stalk hypothesis (Ref: Jahn & Scheller, 2006, Nature Review)

2.9 SNARE Complex Dissociation by NSF and SNAP

Membrane fusion is driven by an adenosine triphosphate (ATP)-dependent cycle of SNARE association and dissociation. In this cycle, the bilayer merger is thermodynamically coupled to exergonic folding of SNARE proteins, followed by their endergonic unfolding mediated by a molecular machinery that dissociates the extremely stable cis v-/t-SNARE complexes residing in a single lipid bilayer. This machinery is composed by the cytoplasmic ATPase, NSF and its adaptor protein, SNAP, the latter binding directly to the SNARE complex (Puri *et al*., 2003). When the N-terminal domain of NSF binds the SNAP/SNARE complex, it dissociates the SNAP/SNARE complex by ATP-hydrolysis and hence dissociates the four-helix SNARE bundle (as shown in figure 8) (Sollner *et al*., 1993a). The ability of NSF to untwist and dissociate SNAREs is called SNARE priming (Klenchin *et al*., 2000). This process is thought to liberate individual SNARE proteins from cis-SNARE complexes (i.e., SNARE complexes present on the same membrane), making them available to form fusogenic trans SNARE complexes between opposing membranes. The resulting individual SNAREs are largely unfolded and remains in an energy-rich state for another round of fusion. In contrast to cis-SNARE complexes, SNAREpins are not substrates for NSF and SNAPs, allowing the stable formation of SNAREpin intermediates (Weber *et al*., 2000).

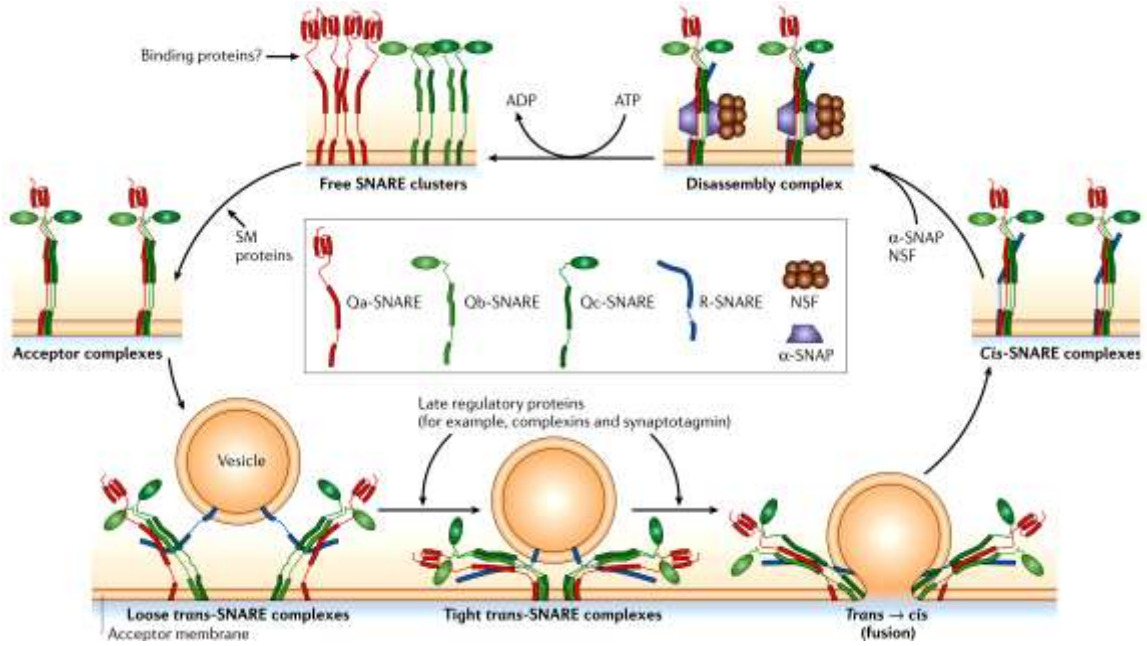


Figure 8: The SNARE conformational cycle during vesicle docking and fusion (Ref: Jahn & Scheller, 2006, Nature Review)

2.10 Regulation of SNARE Activity

SNAREs can assemble spontaneously in vitro; however, in vivo, SNARE complex assembly is rigidly controlled by regulatory proteins, such as Rab GTPases and 11 Sec1 proteins (Rothman and Sollner, 1997). SNARE regulatory proteins can act by preventing inappropriate SNARE complex assembly between cognate v- and t-SNAREs (Pevsner *et al*., 1994; Gaisano HY, 2000). For example, Sec1/Munc18 proteins control the availability of Syntaxins for fusion (Rizo and Sudhof, 2002). When neuronal Munc18a (nSec1) clamps Syntaxin-1 in a certain conformation, it is unable to form complexes with VAMP2 and SNAP-25 (in vitro) (Pevsner J, 1994). With PKC-mediated phosphorylation, Munc18a dissociates from Syntaxin-1 (Fujita *et al*., 1996; Shuang *et al*., 1998), freeing Syntaxin-1 to form SNARE complexes with SNAP-25/VAMP-2 (Pevsner *et al*., 1994; Fujita *et al*., 1996). Mutagenesis experiments, which mutated the closed conformation of Syntaxin-1 (expressed in PC12 cells), made it unable to bind Munc18-1 and decreased secretion (Dulubova *et al*, 1999). From these studies, a model of Munc18-mediated regulation emerges. In this model, Munc18a dissociation causes Syntaxin-1A to undergo a conformational shift from the closed state (which binds Munc18-1) to the open state (which does not bind Munc18-1), thus allowing open Syntaxin-1 to bind other SNAREs and mediate exocytosis (Dulubova *et al,* 1999). Munc18a is also involved in regulating fusion pore dynamics (Fisher *et al*., 2001). One study in a neuroendocrine PC12 cell line

showed that Munc18-1 is also involved in Syntaxin-1 localization to the PM (Arunachalam *et al*., 2008).

Phosphorylation of SNAREs is also an important regulatory mechanism. For SNAP-23 in particular, stimulation of the IgE receptor in mast cells causes IKK2-mediated SNAP-23 phosphorylation, which is involved in regulation of degranulation in mast cells and anaphylaxis (Suzuki and Verma, 2008). Mouse platelet and mast cell degranulation involves SNAP-23 phosphorylation at Ser95 and Ser120 (Hepp *et al*., 2005). In fact, in this particular study, it was found that most of the SNAP-23 involved in SNARE complexes with Syntaxin-4 and VAMP-2 after stimulation was phosphorylated. Additionally, SNAP-23 phosphorylation mutant over-expression caused decreased mast cell exocytosis, further suggesting a role for SNAP-23 phosphorylation in regulated mast cell exocytosis (Hepp *et al*., 2005). Other SNARE regulatory proteins include synaptotagmins and complexins. Syntaptotagmin contains Ca_2^+-binding domains (Sudhof TC, 2004) and it may induce fusion via Ca_2^+-dependent binding to SNAREs and lipids (Tucker *et al*., 2004).

2.11 Protein Targeting

Protein targeting or protein sorting is the mechanism by which a cell transports proteins to the appropriate positions in the cell or outside of it. Sorting targets can be the inner space of an organelle, any of several interior membranes, the cell's outer membrane, or its exterior via secretion.

2.11.1 Targeting Signals

Targeting signals are the pieces of information that enable the cellular transport machinery to correctly position a protein inside or outside the cell. The continuous stretch of amino acid residues in the chain that enables targeting are called signal peptides or targeting peptides. There are two types of targeting peptides,

I. The pre-sequences, and
II. The internal targeting peptides

The pre-sequences are an amino-terminal extension of pre-protein usually of 25-50 residues which can direct non-mitochondrial passenger proteins to mitochondria and across both outer and inner membranes into the matrix. The pre-sequences of different mitochondrial proteins do not show amino-acid sequence identity, but they do have characteristic physicochemical properties. e.g., Tom 20 receptor (Pfanner N, 2000)

To function, these components have to come together on the protein surface by folding. They are called signal patches. In addition, protein modifications like glycosylations can induce targeting.

A signal sequence in membrane proteins, as in secretory proteins, is responsible for the membrane targeting to the endoplasmic reticulum (ER), whereas topogenic sequences (including the signal sequence) determine how the protein is folded in the membrane (Blobel G, 1980). Additionally, it has been shown that N-linked glycosylation of a glycoprotein is also important for the membrane protein folding in the ER (Helenius A, 1994). Membrane insertion and translocation of a nascent membrane protein also involves many translocation-machinery proteins on the ER (Schekman R, 1994).

2.12 Protein Translocation

In 1970, Gunter Blobel conducted experiments on the translocation of proteins across membranes and awarded the 1999 Nobel Prize for his findings. The translation of mRNA into protein by a ribosome takes place within the cytosol. If the synthesized proteins belong to different organelle, they can be transported there in either of three ways, depending on the protein.

2.12.1 Co-translational Translocation

The N-terminal signal sequence of the protein is recognized by a signal recognition particle (SRP) while the protein is still being synthesized on the ribosome. The synthesis pauses while the ribosome-protein complex is transferred to a SRP receptor on the endoplasmic reticulum (ER), a membrane-enclosed organelle. There, the nascent protein is inserted into the Sec61 translocation complex (also known as the translocon) that passes through the ER membrane. The signal sequence is immediately cleaved from the polypeptide once it has been translocated into the ER by signal peptidase in secretory proteins. This signal sequence processing differs for some ER transmembrane proteins.

2.12.2 Post-translational translocation

Even though most proteins are cotranslationally translocated, some are translated in the cytosol and later transported to their destination. This occurs for proteins that go to mitochondria, chloroplasts, or peroxisomes (proteins that go to the latter have their signal sequence at the C terminus). Also, proteins targeted for the nucleus are translocated post-translation. They pass through the nuclear envelope via nuclear pore complex. It is also called gated transport.

2.12.3 Transmembrane Translocation

The amino acid chain of transmembrane proteins, which often are transmembrane receptors, passes through a membrane once or several times. They are inserted into the membrane by translocation, until the process is interrupted by a stop-transfer sequence, also called a membrane anchor sequence. These complex membrane proteins are at the moment mostly understood using the same model of targeting that has been developed for secretory proteins. Seven transmembrane G-protein coupled receptors mostly do not have an amino-terminal signal sequence. In contrast to secretory proteins, the first transmembrane domain acts as the first signal sequence, which targets them to the ER membrane. This also results in the translocation of the amino terminus of the protein into the ER membrane lumen. This would seem to break the rule of "co-translational" translocation which has always held for mammalian proteins targeted to the ER. This has been demonstrated with opsin with in vitro experiments (Kanner *et al*., 2003).

Most mitochondrial proteins are synthesized as cytosolic precursors containing uptake peptide signals. Cytosolic chaperones deliver preproteins to channel linked receptors in the mitochondrial membrane. The preprotein with presequence targeted for the mitochondria is bound by receptors and the General Import Pore (GIP) (Receptors and GIP are collectively known as Translocase of Outer Membrane or TOM) at the outer membrane. The preprotein is translocated through TOM as hairpin loops. The preprotein is transported through the inter membrane space by small TIMs (which also acts as molecular chaperones) to the TIM23 or 22 (Translocase of Inner Membrane) at the inner membrane. Within the matrix the targeting sequence is cleaved off by mtHsp70. Targeting to the outer membrane, inter membrane space, and inner membrane often requires another signal sequence in addition to the matrix targeting sequence.

All **peroxisomal** proteins are encoded by nuclear genes. To date there are two types of known Peroxisome Targeting Signals (PTS):

I. **Peroxisome targeting signal 1 (PTS1):** a C-terminal tripeptide with a consensus sequence (S/A/C)-(K/R/H)-(L/A). The most common PTS1 is serine-lysine-leucine (SKL). Most peroxisomal matrix proteins possess a PTS1 type signal.

II. **Peroxisome targeting signal 2 (PTS2):** a nonapeptide located near the N-terminus with a consensus sequence (R/K)-(L/V/I)-XXXXX-(H/Q)-(L/A/F) (where X can be any amino acid).

2.13 Sorting of SNAREs to Different Compartments

Although SNAREs associated with synaptic vesicle exocytosis is well characterized, very less is known about how these SNAREs are targeted and transported to their resident membranes. SNAREs are integral membrane proteins but do not contain any signal sequence for co-translational insertion into the ER. Syntaxin and synaptobrevin contain a hydrophobic domain at their C-terminus that is responsible for insertion into their resident membranes. Synaptobrevin is post-translationally inserted into the ER membrane by a novel mechanism and is transported to the Golgi where it is sorted to synaptic vesicles (Kutay *et al*., 1995). Syntaxin could potentially follow the same transport route as synaptobrevin, with a specific sorting mechanism from the Golgi to the plasma membrane.

The membrane-binding properties of SNAP-25 are presumed to be due to modification of the protein by palmitate at cysteine residues near the center of the primary amino acid sequence (Hess *et al*., 1992). Indeed, deletion of 12 amino acids, including the four cysteine residues that are the putative sites of palmitoylation of SNAP-25, abolishes incorporation of radioactive palmitate into the mutant protein and results in a protein that is no longer associated with membranes (Veit *et al*., 1996). Therefore, it is likely that palmitoylation of SNAP-25 is required for its association with membranes.

2.14 Microdomains/Lipid Rafts

The lipids that compose cellular membranes are diverse, and as such have different affinities towards proteins and other lipids. The lipid “raft” hypothesis suggests that sphingolipids and cholesterol cluster into discrete regions of the cell membrane (Simons *et al*., 1997). These sphingolipid and cholesterol-rich domains have been termed “lipid rafts” because they exist in a less fluid and more ordered state than glycerophospholipid-rich domains of the membrane. "Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." Lipid rafts are resistant to solubilization by cold nonionic detergents; this resistance has been used as the criterion for raft purification from numerous cell types (London *et al*., 2000). Lipid microdomains or rafts exist in eukaryotic cell membranes and have important functions there (Simons *et al*., 1997). These rafts are likely to be important in the structure and function of caveolae, plasma membrane invaginations that are implicated in signal transduction (Anderson RG*,* 1998), endocytosis (Parton RG, 1994), transcytosis across endothelial cells (Schnitzer *et al*., 1994) and cholesterol trafficking (Smart *et al*., 1996).

Rafts have also been implicated in protein and lipid sorting in the secretory and endocytic pathways (Simons *et al*., 1997).

2.14.1 Lipid Modifications Determine Protein Association with Membrane Rafts

Membrane proteins require specialized structures, which can be either proteinaceous or lipidic, to allow them to embed in the hydrophobic environment of the lipid bilayer. Membrane spanning (or inserting) protein domains are typically α-helices or β-sheets with hydrophobic surfaces serving as the interface to the hydrocarbon core of the lipid bilayer. In addition to, or as a replacement for, hydrophobic protein domains, membrane association can be mediated by lipidic anchors, which can either be permanent co-translational additions or post-translational modifications under dynamic enzymatic control. These lipid modifications include (1) glycophosphatidylinositol (GPI) anchors; (2) N-terminal myristic acid tails; (3) cysteine acylation; (4) isoprenylation; and (5) the addition of C-terminal sterol moieties.

Perhaps the most well characterized examples of lipid modifications determining protein association with membrane rafts are GPI-anchored proteins (GPI-APs). GPI-APs are a widely expressed class of proteins that are involved in a variety of cellular functions including adhesion, membrane trafficking, immune system signaling, and nutrient uptake (Chatterjee and Mayor, 2001; Fujita and Kinoshita, 2009). The GPI-anchor consists of a conserved core oligosaccharide covalently coupled to a phosphoinositide moiety that is embedded in the luminal/exoplasmic leaflet through at least two (sometimes three) glycerol-linked acyl or alkyl chains. This anchor is added into the lumen of the ER to soluble polypeptides, thereby conferring membrane association (Fujita and Kinoshita, 2009).

In contrast to the other common lipid modifications (GPI, myristoylation, and prenylation), S-acylation is the only one that can be dynamically regulated by enzymes, i.e., the lifetime of the modification is shorter than the lifetime of the protein it is modifying. Additionally, significant biochemical evidence points to cysteine modification by saturated fatty acids as a potential mechanism for raft localization for a variety of proteins. Thus, for both soluble and membrane embedded proteins, palmitoylation may act as a dynamically regulated control mechanism determining raft domain association in a variety of physiological contexts.

2.14.2 Association of SNAREs with Microdomains/Lipid Rafts

SNARE proteins association with detergent-insoluble lipid rafts was first documented in polarized Madin-Darby canine kidney (MDCK) cells, where apically targeted SNAREs cofractionated with lipid rafts (Lafont *et al*., 1999). In addition to the analyses of SNARE association with lipid rafts in PC12 cells, more recent work has shown that exocytic SNAREs also interact with detergent-insoluble lipid rafts in 3T3-L1 adipocytes, mast cells, HeLa cells and brain synaptosomes (Chamberlain and Gould, 2002; Pombo *et al*., 2003). Quantitative analysis of multiple experiments showed that 80% of all syntaxin 4 or VAMP-2 bound to SNAP-23 was present in detergent-insoluble membrane microdomains (Puri and Roche, 2006). Most of SNARE complexes containing SNAP-23/syntaxin 4/VAMP-2 are associated with lipid rafts in RBL mast cells. Studies by Puri and Roche, 2006 also shows that syntaxin-4 association with lipid raft microdomains is a consequence of its binding to the raft-localized SNARE SNAP-23.

Membranes should be regarded as lipid-protein composites rather than the often expressed model of a dilute solution of protein in a lipid solvent. Thus, membrane proteins are believed to play an important part in raft formation, and rafts contain many different proteins, including glycerophosphoinositol (GPI)-anchored proteins, tyrosine kinases, phosphatases and other signaling proteins. Palmitoylation or myristoylation can target proteins to lipid rafts, and other proteins with trans-membrane segments are targeted to rafts by specific amino acid sequences. These provide much of the important biological properties of rafts, and are also essential to maintain their stability. The interplay of lipid-based raft units together with protein-mediated assembly of specific protein complexes generates functional domains with high biological activity in cell membranes.

2.14.3 Targeting of SNAREs to Lipid Rafts

Whereas syntaxin and VAMP-2 are anchored to membranes by transmembrane sequences, SNAP-25 and SNAP-23 are synthesized as soluble proteins and become membrane associated via palmitoylation of their respective cysteine-rich domains (Veit *et al*., 1996; Vogel *et al*., 1999). Palmitoylation is a post-translational modification of proteins that involves the attachment of the C16 saturated fatty acid palmitate most often to cysteine residues via a thioester linkage i.e., S-palmitoylation (Greaves *et al*., 2009b). The majority of cellular palmitoylation events are enzyme-mediated, and recent work identified a family of 23 DHHC proteins that function as palmitoyl transferases (Fukata *et al*., 2004). The defining feature of DHHC proteins is a 51-amino acid domain containing a DHHC motif (aspartate-histidine-histidine-cysteine) within a cysteine-rich

(CR) domain. This DHHC-CR domain is thought to contain the catalytic site of DHHC proteins. SNAP-25B is palmitoylated by DHHC3, DHHC7, and DHHC17 (Fukata *et al*., 2004; Greaves *et al*., 2009a). There is little information available on the enzymes that modify SNAP-25a and SNAP-23.

Palmitoylation is a major raft-targeting signal, and around 50% of detergent-insoluble raft proteins in MDCK cells could be labeled with palmitate (Melkonian *et al*., 1999). SNAP-25 is multiple palmitoylated and this probably accounts for its accumulation in lipid raft domains. This is suggested because the SNAP-25 homolog SNAP-23 is also palmitoylated but yet is present in raft fractions at 3-fold higher levels than SNAP-25 (Chamberlain and Gould, 2002; Pombo *et al*., 2003). However, in addition to the cysteine rich domains of SNAP-25/23, a short stretch five-amino acid motif (^{116}QPARV^{120}) at the C-terminus of the domain is also essential for palmitoylation and membrane binding (lipid raft association) of SNAP-25B (Gonzalo *et al*., 1999; Greaves *et al*., 2009a). Mutation of three of the five amino acids (Gln, Pro, and Arg) to alanine severely compromised localization of the membrane-targeting domain and significantly decreased palmitate incorporation into SNAP-25. SNAP-25 is synthesized as a soluble protein, but must associate with a membrane-bound palmitoyltransferase to become fatty acylated (Gonzalo *et al*., 1998). This sequence is identical in SNAP-25B but only partially conserved in SNAP-23 (mouse sequence is QPSRI). Mutation of this region in SNAP-25B inhibits membrane binding and plasma membrane targeting in PC12 cells (Gonzalo *et al*., 1999; Greaves *et al*., 2009a). Though Palmitoylation is required for initial membrane targeting of SNAP-25, but once at the membrane, its interaction with other SNARE proteins like Syntaxin and VAMPs can preserve membrane association in the absence of palmitoylation (Gonzalo *et al*., 1999). SNAP-23 relocates in response to stimulation from plasma membrane lamellipodia-like projections to granule membranes in permeabilized mast cells. While relocation is a prerequisite for secretion, it can occur without membrane fusion and will expedite a subsequent secretory response. After relocation, SNAP-23 is required for exocytosis, implying a crucial role in promoting membrane fusion (Guo *et al*., 1998).

Whereas SNAP-25/23 contain readily identifiable putative raft-targeting signals, the mechanism underlying syntaxin accumulation in rafts is not obvious. Syntaxin 1A has been shown to interact with cholesterol (Lang *et al*., 2001), providing a potential mechanism for raft accumulation of this protein. However, a recent study demonstrated that syntaxin 1A was largely excluded from sphingolipid/cholesterol-rich domains in model membranes (Saslowsky *et al*., 2003), suggesting that syntaxin 1A may not have an intrinsic affinity for lipid rafts, and that raft accumulation of this protein depends upon its interaction with another raft protein. Indeed, syntaxin 1A and SNAP-25 have recently

been shown to extensively co-distribute in clusters at the PM of chromaffin cells. This co-clustering was abolished by disrupting the syntaxin/SNAP-25 interaction, suggesting that the microdomain localization of these SNAREs is dependent upon their interaction with each other (Rickman *et al*., 2004).

3 MATERIALS AND METHODS

3.1 Cell Lines

3.1.1 HeLa Cell Lines

Cell type: Human cervix carcinoma
Origin: Taken from cervix Carcinoma of a 31 year Henrietta Lacks in 1951
Morphology: Epithelial-like cells growing in monolayer

Hela cells (Ref: National Center for Cell Science, Pune) are an adherent cell line i.e., they stick to the bottom of the cell culture flask. They are able to rapidly grow till the cells come in contact with each other and then they stop growing. This growth pattern is a classic growth pattern of oncogenic cells. Both DMEM and RPMI 1640 growth medium can be used to culture HeLa cells. The doubling time for Hela cells are approximately 24 hours.

3.1.2 A549 Cell Lines

Cell type: Human Lung Carcinoma
Origin: Established from an explanted lung tumor which was removed from a 58-year-old Caucasian man in 1972
Morphology: Epithelial cells, growing adherently as monolayer
Cytogenetic Information: The cell line is hypotriploid with a modal chromosome number of 66, which occurs in 24% of cells.

A549 cells (Ref: National Center for Cell Science, Pune) are adenocarcinomic human alveolar basal epithelial. These are squamous in nature and responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs. They grow adherently, as a monolayer, and cultured in Dulbecco's MEM with 10% FBS. The doubling time for A549 cells are approximately 40 hrs.

3.2 Bioinformatics Approach for the Generation of Fusion Proteins

A bioinformatic approach (as shown in figure 9) was followed for identification of potential transmembrane protein that has multi-spanning domains, and not having the targeting sequence for lipid rafts. The search was performed by using the following protein databases.

I. The European Bioinformatics Institute databases
   - UniProt
   - UniProtKB/Swiss-Prot
   - InterPro

II. The **ExPASy** (Expert Protein Analysis System) proteomic server
   - UniProt Knowledgebase (Swiss-Prot and TrEMBL)

III. **NCBI** (National Center for Biotechnology Information) - Protein Database

This broad search yielded a number of potential target proteins. These proteins were studied in detail for its membrane topology, functions, sequence analysis, post translational modifications, tissue specific expression by using following online analysis tools and the search was first narrowed down to a few potential protein candidates and then narrowed down to a few transmembrane domains from these proteins.
Given below is a brief description of the tools used for protein analysis:

I. ExPASy Proteomics Server:
(http://www.expasy.org/links.html)

Various online Post translational modifications prediction tools enlisted below were used for analysis of post-translational modification in the polypeptide sequences. These online tools were made available by ExPASy proteomics server.

a. GPI-SOM:
(http://gpi.unibe.ch/)

This online tool was used for identifying any glycosylphosphatidylinositol (GPI) anchors in the protein. Cell surface proteins can be attached to the cell membrane via the glycolipid structure called glycosylphosphatidylinositol (GPI) anchor. It is assumed that GPI-anchor is also responsible for taking the protein to the raft region of the membrane. The transmembrane domains which showed the probability of getting GPI anchor in them were neglected.

b. NMT-The MYR Predictor:
(http://mendel.imp.ac.at/myristate/SUPLpredictor.htm)

This online tool was used for predicting myristoylation in the polypeptide sequence. In myristoylation a myristoyl group (derived from myristic acid) is covalently attached via an amide bond to the alpha-amino group of an N-terminal amino acid of a nascent polypeptide chain that is responsible for signaling proteins to the raft region of the membrane. Only those

transmembrane domains were selected that did not show any myristoylation on them

c. CSS-Palm 3.0:

This software was used to predict the site of palmitoylation in the polypeptide chain. Contrasting to the labor-intensive and time consuming experimental approaches, in-silico prediction of palmitoylation by CSS-Palm 3.0 is a popular strategy (Ren *et al*., 2008). Plamitoylation is an essential post-translational modification in which fatty acids, such as palmitic acid, is covalently attached to cysteine residues of membrane proteins. This is also responsible for increasing the hydrophobicity of a protein and its interaction with membrane. So only those transmembrane domains were selected that did not show any palmitoylation in them.

II. GeneNote (Gene Normal **T**issue **E**xpression**):**
(http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/home_page.pl**)**

GeneNote is a database of human genes and their expression profiles in healthy tissues. It is based on Weizmann Institute of Science DNA array experiments, which were performed on the Affymetrix HG-U95 set, which includes 62 839 probe-sets. The hybridization intensities of two replicates were processed and analyzed to yield the complete transcriptome for twelve human tissues (Shmueli *et al*., 2003). It offers an expression profile for each gene in the human genome. Expression pattern of potential protein candidates were identified in different human tissues as well as cancer cell lines using the above mentioned databases. The information helped us to identify the potential tissues (cell lines) that could be used for generating the desirable nucleotide sequence by RT-PCR followed by PCR. The tissues that showed higher level of desirable gene expression were selected for RNA isolation.

III. Transmembrane Protein Topology Prediction Server (TMMOD):
(http://liao.cis.udel.edu/website/servers/TMMOD/scripts/frame.php?p=submit)

TMMOD is an online server for transmembrane proteins topology prediction using a hidden Markov model. TMMOD uses TMHMM as a prototype, but differs from TMHMM by the architecture of the submodels for loops on both sides of the membrane and also by the model training procedure (Kahsay *et al*., 2005). TMMOD has a higher accuracy in topology and location prediction and fewer false positive as compared to TMHMM. This online modeling tool was used to predict the topology of the fusion proteins which will be generated using our approach.

IV. National Center for Biotechnology Information:
(http://www.ncbi.nlm.nih.gov/)

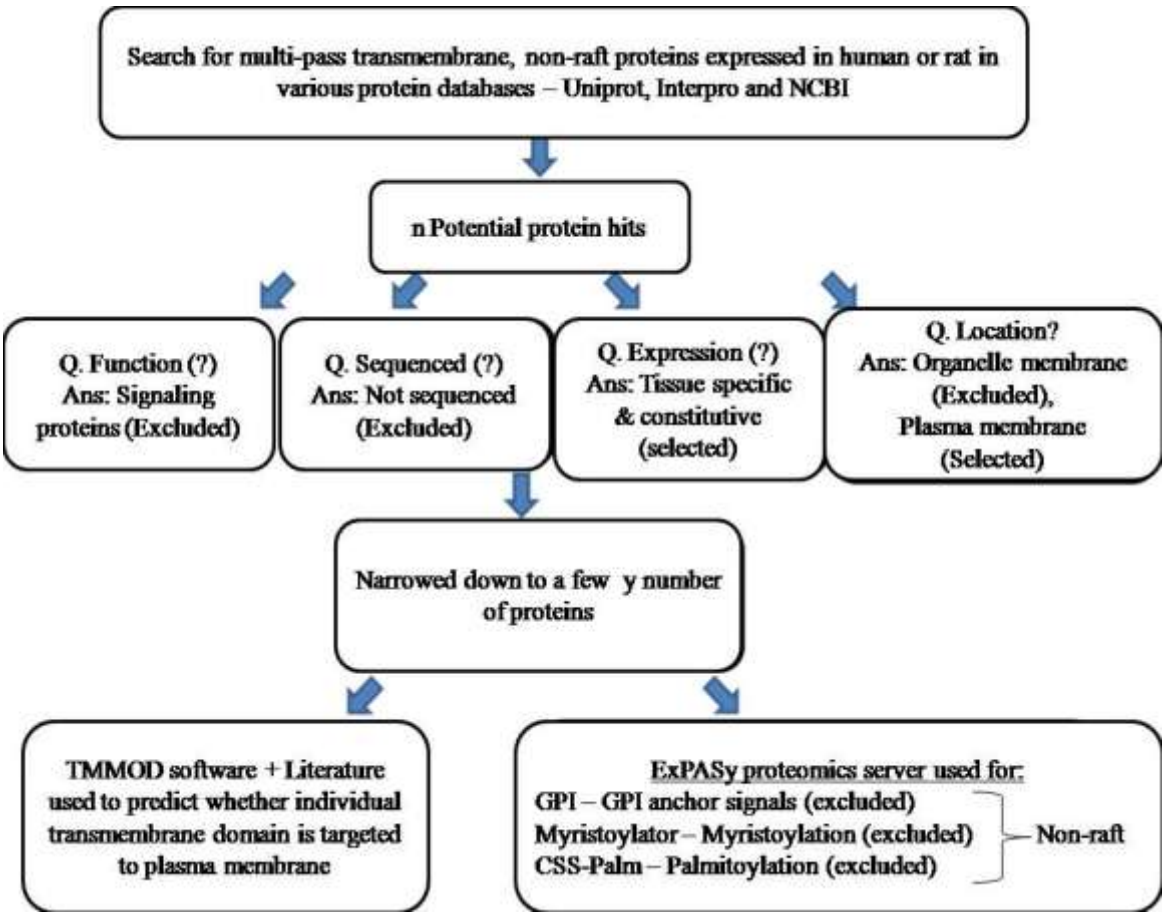


Figure 9: Flow chart of Bioinformatic approach for selecting transmembrane domain from different membrane proteins

3.3 Designing of Primers

The primers were designed manually for MDR3 and (1-145 amino acid residues), MDR3 (41-145 amino acid residues) and CLP24 (134-195 amino acid residue) using the guidelines for primer designing and were then tested for various parameters like length of primer, GC content, Tm, self complementarities, hairpin formation, etc., using tools enlisted below. The primers were designed in such a way that it contains restriction enzyme site and does not shift the reading frame of the fusion protein. Another primer was designed for human GAPDH (hGAPDH) gene using Primer-BLAST-NCBI and the product length was adjusted to 452bp. This hGAPDH primer was used for checking the quality of cDNA synthesized (positive control).

Table 1: List of primers used

Name	Sequence	Length	GC %	Tm (^{0}C)	
				Basic	Salt Adjust
MDR3 Sense 1	5'GCGCGACAAATGGTCAGATGGATCTTGAGGCG 3'	32	56 %	67	76.2
MDR3 Sense 41	5'GCGCGACAAATGGTCAGGGAGTATTAACATTG 3'	32	47 %	63	72.5
MDR3 Antisense 145	5'GCGCGACCATTTGTCTGTCGACCAGCTGC 3'	29	62 %	67.2	76
CLP24 Sense 134	5'GAGCGACAAATGGTCAGTGGGAGGAGGCCATGGC 3'	34	62%	70.4	80.2
CLP24 Antisense 195	5'GCGCGACCATTTGTCTCCCTCTTGTGGAGAATG 3'	33	55 %	66.9	76.6
hGAPDH Sense	5' ACCACAGTCCATGCCATCAC 3'	20	55 %	54.4	
hGAPDH Antisense	5' TCCACCACCCTGTTGCTGTA 3'	20	55 %	55.0	

Key:

ACGT – Kozak Sequence
ACGT – Ahd I Site
ACGT – Extra Nucleotides (to maintain reading frame)
ACGT – Nucleotide Sequence/complementary to gene sequence
ACGT – Common (between Ahd I Site and Gene Sequence)

3.3.1 Tools Used for Designing Primers

a. **Oligo Calc:** (Oligonucleotide Properties Calculator) (http://www.basic.northwestern.edu/biotools/oligocalc.html)

b. **Integrated DNA technologies; Oligo analyzer 3.1** (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/)

c. **Primer-BLAST:** (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

d. **MultAlin:** (Multiple sequence alignment by Florence Corpet) (http://multalin.toulouse.inra.fr/multalin/)

3.4 Preparation of Competent Cells

Competent *E. coli* cells were created 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^{o}C. Liquid culture in LB medium was initiated from a single colony and kept in shaker/incubator at 37^{o}C for overnight. Overnight grown cells were used as inoculums to obtain culture at log phase. *E. coli* was grown in 50 ml flask till the optical density at 600 nm reached 0.40-0.45. After 15 min of vigorous shaking on ice-cold water, the cells were collected by centrifugation at 4700 rpm for 15 min at 4^{o}C. The pellet was resuspended in 25 ml of fresh acid salt buffer containing 100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM Sodium acetate (pH 5.2), and incubated on ice for 40 min. The suspension was centrifuged at 4700 rpm for 10 min and the pellet was resuspended in 1 ml of acid salt buffer. The competent cells were stored as 15% glycerol stocks in small aliquots at -70^{o}C.

3.5 Transformation of Competent *E. coli* with Plasmid DNA Construct

The transformation was carried out as described by Sambrook and Russel, 2001. Briefly, 50 ng of DNA was added to 75 µl *E. coli* DH5α in a 1.5 ml pre-chilled tube on ice and the, incubation continued for 15 min after gentle mixing by pipetting up and down a few times. After that, a brief "heat shock" was given at 42°C for 60 sec in water bath and the cells were immediately chilled on ice for 2 min. To this *E. coli*-DNA mix, 925 µl SOC medium was added and cells were allowed to grow at 37^{o}C for 1 hr in a shaker incubator at 225 rpm. The *E. coli* cultures were centrifuged at 10,000 rpm for 5 min and 925 µl of the supernatant was removed. The *E. coli* cell pellet was resuspended in the remaining

75 µl SOC medium. It was plated onto the kanamycin (50 µg/ml of media) containing LB agar plate and incubated overnight at 37°C. Small colonies of *E. coli* that were able to grow on media after incubation would have been transformed by the plasmid construct. The efficiency of the transformation was calculated by using the following formula.

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

3.6 Maxipreparation of Plasmid DNA (by Alkaline lysis method)

3.6.1 Harvesting of Bacterial Cells

A single colony of *E. coli* (DH5-α) cells containing the desired plasmid construct was picked up and inoculated to 4ml LB media containing kanamycin (50 µg/ ml of media) in a 15 ml falcon tube and grown overnight at 37ºC with shaking at 220 rpm. 1 ml of this primary culture was transferred into 15 ml LB media containing kanamypcin in a 50 ml falcon tube and grown for 4-6 hours at 37^{o}C with shaking at 220 rpm. 30 ml of this secondary culture was transferred into 500 ml LB media containing kanamycin in a 2 liter conical flask and grown for 12-16 hours at 37^{o}C with shaking at 220 rpm. Culture was then transferred to GSA bottles and cells harvested by centrifugation at 6,000 rpm for 15 minutes at 4°C in Hitachi RIOA3 rotor. Supernatant was drained off and the pellet was resuspended in 50 ml of ice cold STE solution. The solution was centrifuged at 6000 rpm for 15 min at 4°C.

3.6.2 Alkaline Lysis

The pellet was resuspended in 18 ml of ice cold solution I with vigorous shaking and then 20 µl of RNAse (10 mg/ml) was added. Thereafter, 18 ml of freshly prepared solution II was added and mixed gently by inverting the GSA bottles 10 times and then incubated at room temperature for 10 min. Then 40 ml of ice cold solution III was added and mixed thoroughly by inverting the tubes for 10 times and was incubated on ice for 10 min. Cell debris was removed by centrifugation at 9,000 rpm for 40 min at 4°C and the supernatant was filtered through two layers of cheese cloth. 0.6 volume of isopropanol was added to the filtrate, mixed by inversion and then incubated at room temperature for 10 minutes. Tubes were then centrifuged at 9,000 rpm for 20 min at room temperature. The pellet was washed with 70% ethanol and then centrifuged at 9,000 rpm for 7 min. The DNA pellet was allowed to air dry and then resuspended in 50 µl of TE buffer at 37^{o}C and stored at -20^{o}C.

3.6.3 Determination of Concentration and Purity of DNA Using Nanodrop ND3000 Spectrophotometer

DNA samples were quantified by using Nanodrop ND3000 Spectrophotometer. The purity of the DNA sample was calculated by taking a ratio between the readings at 260 nm and 280 nm.

DNA purity ratio = A_{260}/A_{280}

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

Pure preparation of DNA has OD_{260}/OD_{280} values of 1.8, preparation of DNA having RNA as impurity has OD_{260}/OD_{280} values greater than 1.8 while samples having proteins have OD_{260}/OD_{280} values less than 1.8. The absorbance at 260 nm provides an estimate of the concentration of the nucleic acid in the sample.

Concentration of Plasmid DNA = 50 x A_{260} nm x dilution factor

Where, A_{260} = optical density of Plasmid DNA determined at 260 nm. An OD_{260} of 1 corresponds to approximately 50 µg/ml of DNA.

3.6.4 Restriction Digestion of Plasmid DNA

1µg DNA sample was placed in a sterile microfuge tube and mixed with 14 µl of MQ H_2O. 2 µl of appropriate 10X restriction enzyme digestion buffer (BamHI), 2 µl (10 units/µl) restriction enzyme (BamHI from FERMENTAS) were added to volume make upto 20 µl and mixed by tapping the tube. The sample was incubated at 37^{o}C in water bath for 4 hrs.

- **Agarose gel electrophoresis for digested plasmid DNA:**

1.8 % agarose gel (100 ml) was prepared in 0.5X TBE buffer and 1.5 µl (0.15 µg/ml) of ethidium bromide was added to it. Gel cassette was prepared and DNA samples were loaded along with the 6X gel loading dye. Voltage (90 Volts) was supplied for 2 hours and the gel was observed under Biorad GELDOC System.

3.7 Maintaining HeLa Cell Culture

HeLa cells were cultured invitro and used for isolating RNA for RT PCR. The cells were maintained by subculturing in fresh RPMI medium containing 10 % fetal bovine serum (FBS), 25mM HEPES and 120 µg/ml gentamycin (RPMI complete medium) at 37^{o}C &

humidified atmosphere containing 5% CO_2. The media was discarded from T-25 flask containing HeLa cells culture and washed for 2 times by adding prewarmed PBS gently along the walls. Then 1ml prewarmed PBS and 30 µl Trypsin (0.25 % w/v trypsin in 10mM EDTA) was added and incubated at 37^{o}C for 4 min. At the end of this incubation, the cells were detached from the walls of T-25 flask and were harvested in 4 ml RPMI-CM by pipetting. The culture was transferred to a sterile falcon tube, sealed and then centrifuged at 1500 rpm for 5 min at 4^{o}C. The supernatant was aspirated and pellet was washed with PBS and then again centrifuged as above. The pellet was then resuspended in 1 ml of RPMI-CM. The cells were counted under Haemocytometer and viability determined after staining with trypan blue (Sigma Aldrich). Cells were then seeded at an appropriate cell number in 5 ml media in T-25 culture flask and incubated at 37^{o}C in humidified atmosphere containing 5% CO_2.

3.7.1 Determination of Cell Count and Viability Using Haemocytometer

Haemocytometer is a thickened glass slide having a small chamber of grids cut into the glass. The chamber is etched in 9 large squares each measuring 1mm x 1mm in area and 0.1mm in depth. With the coverslip in place each square represents a volume of 0.1 mm^3. Cell count can be estimated using the formula:

Cells/ml = Number of cells counted x **dilution factor** / **volume of sample (in ml)**

Trypan Blue is called a vital dye as it is negatively charged and does not interact with the cell unless the membrane is damaged (Freshney RI, 2010). Therefore, all the cells which exclude the dye appear as bright translucent structures and are viable whereas the dead cells stain blue. This principle is used to ascertain the viability of cells. The number of viable cells when expressed in percentage is called as viability and can be given by the formula:

$$\text{Percentage viability} = \frac{\text{Number of viable cells counted}}{\text{Total number of cells counted}} \times 100$$

3.7.2 Percentage Viability of HeLa Cell Culture

HeLa cells were cultured in a 48 well culture plate. 20,000 cells were seeded/ ml of RPMI-CM/ well in 14 wells in duplicate of 4 different time points (i.e., 6 hrs, 12 hrs, 24 hrs and 36 hrs). For this 0.16x10^6 cells were suspended in total of 8 ml RPMI-CM and then 1/1 ml was dispensed in each of 8 wells. The plate was incubated at 37^{o}C in

humidified atmosphere containing 5% CO_2. After each time points mentioned above, total cells were harvested from two wells separately for the mentioned time point by trypsinization and thorough washing of the wells with prewarmed PBS. The harvested cells were then stained with trypan blue and counted for dead and live cells on Haemocytometer. Percentage viability was then calculated.

3.8 RNA Isolation (from HeLa and A549 Cells)

3.8.1 Precautions and Preparations Required for RNA Isolation (In order to avoid RNAse action)

0.1 % v/v DEPC (Diethyl Pyrocarbonate) treated water was prepared by adding 2 ml DEPC in 2 liter of MQ H_2O covered with aluminium foil and stirred overnight at 200 rpm. Pipette tips, eppendorf tubes, falcon tubes and glasswares were treated with DEPC treated water for 10-16 hrs at 37^oC as described by Sambrook and Russel, 2001 and then sterilized by autoclaving. Electrophoresis tank was also treated with DEPC water for overnight. Precautions (wearing gloves and lab coats, cleaning of the working area with DEPC treated water, etc.) were taken while performing experiments to avoid RNAse contamination.

3.8.2 Sample Preparation

Cells were harvested by centrifugation process at 1200 rpm for 15 min. 1 ml of TRI reagent (Sigma) was added to 10×10^6 HeLa cells in an eppendorf tube and the cells were lysed by repeated pipetting. The solution was allowed to stand for 5 minutes at room temperature. 0.2 ml of chloroform was added per ml of TRI reagent used and vortexed for 15 sec. The solution was allowed to stand for 15 minutes at room temperature. The tube was then centrifuged at 12000×g for 15 min at 4^oC (Chomczynski and Mackey, 1995).

4.8.3 RNA Isolation

Three distinct phases were seen in the eppendorf tube. The upper aqueous layer was transferred into a fresh eppendorf tube by careful pipetting and 0.5 ml of isopropanol per ml of TRI reagent was added to the tube. It was mixed well, allowed to stand for 15 min at room temperature and then centrifuged at 12000×g for 10 min at 4^oC. The supernatant was discarded carefully and RNA pellet was washed with 1ml of 75% ethanol and centrifuged at 12000×g for 7 min at 4^oC. The obtained RNA pellet was air dried and dissolved in 30 µl of DEPC treated water at room temperature and then 1µl

(40u/µl) RiboLock RNase Inhibitor (Fermentas) was added. RNA was isolated from different batches of cells with varying cell counts in order to determine the RNA recovery.

- **Determination of concentration and purity of RNA using Nanodrop ND3000 Spectrophotometer:**

  The purity of the RNA sample was calculated by taking a ratio between the readings at 260nm and 280nm. Pure preparation of RNA had OD_{260}/OD_{280} values of ≥1.7

- **Formaldehyde agarose gel electrophoresis for RNA**

1.2 % formaldehyde agarose gel was prepared by boiling 1.2 gm agarose powder in 100 ml of 1X formaldehyde agarose gel running buffer and 1.5 µl (0.15 µg/ml) ethidium bromide was added after cooling to 60-70^{o}C. Thereafter the gel was casted and was put into the gel tank containing 1X Formaldehyde agarose gel running buffer. RNA samples (1 volume 5X RNA loading dye + 4 volume of RNA) were loaded along with the standard RNA ladder (NEB, UK). Voltage (90 V) was supplied for 2 hours and the gel was visualized under Biorad GELDOC System.

3.9 Reverse Transcriptase PCR

5 µg of isolated RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase enzyme (Promega) in a reaction mixture of 25 µl. Reaction mixture was prepared in 0.2 ml PCR tube by mixing nuclease free MQ H_2O (7µl), M-MLV RT 5X buffer (5 µl), RNA template (3 µl) and oligo(dT) primer (2.5µl) (Fermentas). The reaction mixture was kept on water bath for 10 min at 70^{o}C and thereafter immediately kept on ice. 6 µl dNTPs mix (Promega) and 1.5 µl (200 u/µl) M-MLV reverse transcriptase was added to the mixture.

Following reaction condition was used:

30^{o}C for 10 min
37^{o}C for 90 min
72^{o}C for 10 min
4^{o}C for ∞

The cDNA was run onto 1.2% agarose gel containing ethidium bromide (0.15 µg/ml) for 2 hours at 90 volts and then visualized under Biorad GELDOC System.

3.10 Gradient PCR for Human GAPDH Gene

Gradient PCR was performed for human GAPDH gene amplification using the cDNA as template. Reaction mixture was prepared for five PCR reactions 25 µl each. The components were mixed in a PCR tube as shown in the table below and then gradient PCR was set at five different temperatures.

Table 2: Reaction components for gradient PCR for hGAPDH gene

Components	**Stock Conc.**	**Final Conc.**	**Volume/ reaction**
MilliQ H_2O	-	-	15.4 µl
Std. Taq reaction buffer	10X	1X	2.5 µl
dNTPs	100 mM	2.4 mM	0.6 µl
Forward primer	10 µM	0.2 µM	0.5 µl
Reverse primer	10 µM	0.2 µM	0.5 µl
cDNA	-	-	5.0 µl
Taq DNA polymerase	5000 U/ml	100 U/ml	0.5 µl
Total volume			**25 µl**

The PCR conditions were as follows:

Step 1: 95°C for 30 sec
Step 2: 95°C for 30 sec
Step 3: 50°C
gradient
for 1 min
54°C
} X 40 cycles
Step 4: 68°C for 1 min
Step 5: 68°C for 10 min
Step 6: 4°C for ∞

1.8% agarose gel containing ethidium bromide (0.15µg/ml) was prepared in TBE buffer. Gel loading dye was added to the PCR products and mixed well by pipeting. The reaction mixture was loaded onto gel along with 1 µl of 100 bp DNA ladder from NEB, UK (500 µg/ml) and run for 2 hours at 90 V. The gel was visualized under Biorad GELDOC System.

- (5 µl 6X gel loading dye + 25 µl PCR reaction mixture)
- (4 µl gel running buffer + 1 µl DNA ladder + 1 µl 6X gel loading dye)

3.11 PCR for MDR3 (AA, 1-145) and CLP-24 (AA, 134-195) Using QIAGEN OneStep RT-PCR kit

Reverse transcription and PCR was carried out sequentially in the same tube. All components required for both reactions were added during setup. Template RNA, primer solution, dNTP Mix, 5X QIAGEN OneStep RT-PCR buffer and RNase-free water were thawed and placed in ice. A master mix was prepared according to the table given below. The master mix contains all the components required for RT-PCR except the template RNA. After preparation of master mix it was mixed thoroughly by pipeting few times up and down and then appropriate concentration of template RNA was added to the PCR tube containing master mix and then One-Step RT-PCR was run in a thermal cycler according to the program outlined below. The tube was placed into the block only after the thermal cycler had reached 50^{o}C.

Table 3: Reaction components for one-step RT-PCR

Components	**Vol/reaction**	**Final Conc.**
Master Mix		
RNase-free water	14.3 µl	-
5X QIAGEN OneStep RT-PCR Buffer (with 12.5mM $MgCl_2$)	5.0 µl	1X
dNTP Mix (containing 10 mM of each dNTP)	1.0 µl	400 µM of each dNTP
Sense Primer	1.5 µl	0.6 µM
Antisense Primer	1.5 µl	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 µl	-
RNase inhibitor	0.2 µl	5 unit/reaction
Template RNA	0.7 µl	2 µg
Total Volume	**50.0 µl**	**-**

Thermal cycler conditions:

Reverse transcription:	50^{o}C	30 min	
Initial PCR activation step:	95^{o}C	15 min	
Denaturation:	94^{o}C	1 min	X 40 cycles
Annealing:	48^{o}C (for MDR3 AA, 1-145)	1 min	
	50.1 ^{o}C; 53.2 ^{o}C; 51.7 ^{o}C;		
	52.4 ^{o}C; 50.6^{o}C (for CLP24 AA, 134-195)	1 min	
Extension:	72^{o}C	1 min	
Final Extension	72^{o}C	10 min	

The PCR products were run onto 1.8 % agarose gel along with 100 bp DNA ladder at 90 V for 90 minutes and visualized under UV-light.

4. RESULTS

4.1 Bioinformatics Approach

Literature alienation: 50 proteins were selected that were associated with the terms "human OR rat AND multi AND membrane-spanning NOT lipid-rafts" in interPro, UniProt, ExPASy and NCBI protein databases. In this set of 50 proteins, 30 proteins were extracted by utilizing InterPro, 10 proteins by UniProt and 5 from NCBI and ExPASy protein databases. Among them 10 proteins were common which is shown in the Venn diagram of figure 10. These proteins were membrane spanning which belonged to human and did not show any association with membrane raft. The common proteins were selected on the basis of their complete structural details, individual work on membrane targeting domains, does not participate in any signaling, function and expression of these proteins in different tissues. Finally this search yielded two membrane proteins that were found to be potential candidate for using in generating fusion protein.

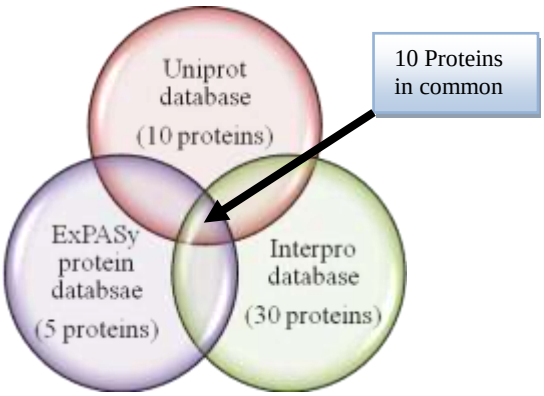


Figure 10: Diagrammatic Representation of literature mining for transmembrane proteins by different protein databases.

4.1.1 Human Membrane Glycoprotein P (MDR3)

Recommended name: Multidrug resistance protein 3
Alternative name(s): ATP-binding cassette sub-family B member 4, P-glycoprotein 3
Organism: *Homo sapiens*
Sequence length: 1286 AA
Sequence status: Complete
Sub cellular location: Cell membrane, multi-pass membrane protein

The human MDR3 gene is a member of the multidrug resistance (MDR) gene family. MDR3 P-glycoprotein is a transmembrane protein that translocates phosphatidylcholine. It is a multi drug resistance protein of 140 KDa ATP dependent plasma membrane associated protein showing two transmembrane domains each composed of six helices (where transport substrates are recognized) and two ATP utilizing sites (where ATP is hydrolyzed) as shown in figure 11 (Orlowski *et al*., 2006). A set of different regulating phosphorylation sites ('P', star) and the glycosylated moiety branched on the first extracellular loop are shown in the figure 2 Independent hydrophobicity analysis using the methods of Kyte-Doolitte (Gros *et al*., 1986b) and Eisenberg (Chen *et al*., 1986) predict a polypeptide which traverses the plasmamembrane 12 times. A cluster of potential N-linked glycosyaltion sites are consistently located in the first external loop. The molecular functions include protein binding, hydrolase activity, ATPase activity coupled to transmembrane movement of substances, ATPase activity, nucleotide binding, xenobiotic-transporting.

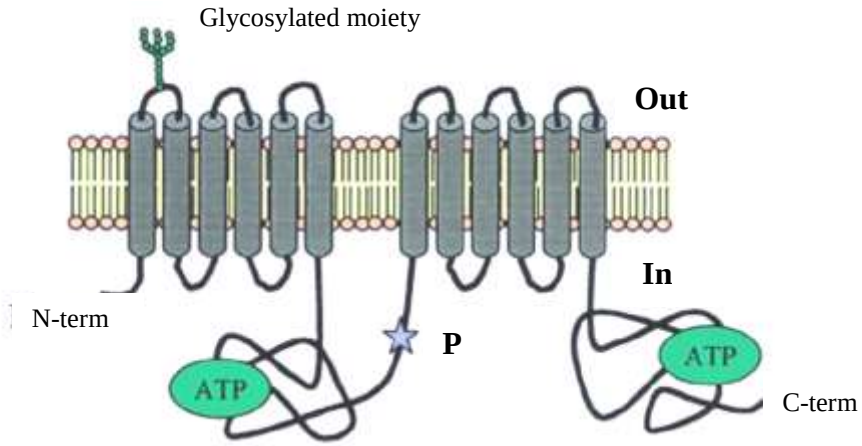


Figure 11: Secondary structure prediction of human MDR3 Pgp

4.1.2 Transmembrane Protein 204 (CLP24)

Recommended name: Transmembrane protein 204
Alternative name(s): Claudin-like protein 24
Organism: *Homo sapiens*
Sequence length: 226AA
Sequence status: Complete
Subcellular location: Cell junction, adherens junction. Cell membrane, Multi-pass membrane protein.

The CLP24 protein contains four predicted transmembrane domains and a C-terminal protein–protein interaction domain. These domains are characteristic of the four transmembrane spanning (tetraspan) family and are involved in cell adhesion at tight, gap and adherens junctions. The protein product of the CLP24 gene contains four transmembrane spanning domains together with a C-terminal protein–protein interaction. The molecular functions include cell-cell interactions through adherens and paracellular permeability. Figure 12, is a schematic representation of the tetraspan structure of the CLP24 protein with four transmembrane domains, a C-terminal protein–protein interaction domain(PDZ domain), and a characteristic extracellular loop structure (Kearsey *et al*., 2004). (PDZ) domain is a hypoxically regulated adherens junction component that is able to influence vascular permeability.

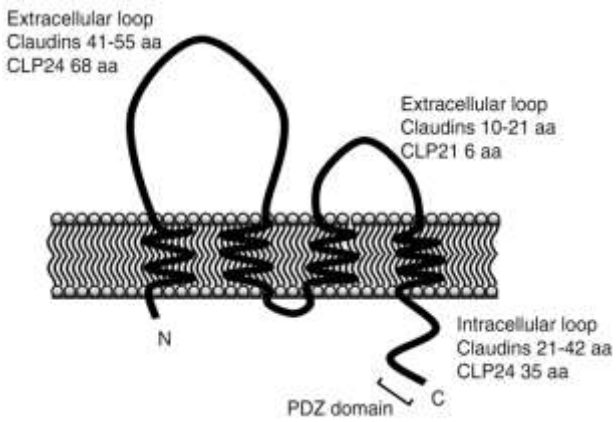


Figure 12: Membrane topology model of Claudin-like protein of 24 kDa (CLP24)

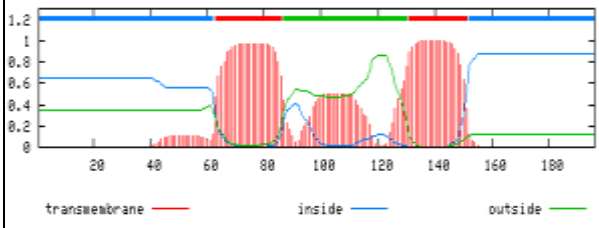
4.2 Membrane Topology and Post-translational Modifications Prediction of Different Polypeptide Sequences

According to the involvement of proteins in mechanism of membrane fission and fusion in the exocytotic and endocytic pathways, it was clear that "Membrane topology" and "Post-translational modifications" were identified as most significantly enriched, with the proteins assigned to this, respectively. In total, five different processes were involved. Among them the first one was the structural studies of each protein, by using literature mining. The next was topology prediction through bioinformatic analysis by an online analysis tool called **'TMMOD'**, which was based on the Hidden Markov Model.

The results shown in the table 4 and 5 are obtained from the server, TMMOD, UniProt and different post-translational modification prediction tools. Table 4(a), (b), (c), (d), (e) contains the information for different transmembrane domains of **Human P-Glycoprotein (MDR3)** and table 5(a), (b) contains information for **Claudin Like Protein 24.**

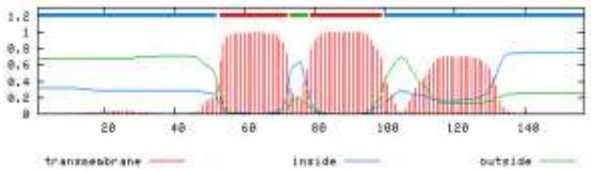
- Column 1 displays about the protein name.
- Column 2 displays the protein sequence obtained from UniProt that was used as input data in the TMMOD server. The polypeptide sequence contained at least two transmembrane domains with one extracellular loop and both N- and C-terminal in the cytoplasm.
- Column 3 displays the output information from TMMOD server. It contains information about the length (number of amino acid (AA) residues) of the protein sequence, number of transmembrane (TM) helices, number of AAs present in each TM helix (if greater than 18 then it was considered as TM protein), number of first 60 amino acids present in the TM helix (if more than few then it was considered that N-terminal of TM helix had signal peptide), total probability of AAs in cytoplasmic side of membrane present in the protein sequence and detailed account of AAs related to the inside, TM helix, outside to the membrane. The figure in the table is the graphical representation of inside, TM helix, outside of the submitted protein sequence.
- Column 4, 5 and 6 displays the results for possible post-translational modifications that could be present on the polypeptide sequence. This includes Myristoylation(MYR), Palmitoylation(PALM) and GPI-anchor signals(GPI).

Table 4(a): Human P-Glycoprotein (AA, 1-188)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (1-188) MDLEAAKN GTAWRPTSA EGDFELGISS KQKRKKTKT VKMIGVLTLF RYSDWQDKL FMSLGTIMA IAHGSGLPL MMIVFGEM TDKFVDTAG NFSFPVNFSL SLLNPGKILE EEMTRYAYY YSGLGAGVL VAAYIQVSF WTLAAGRQI RKIRQKFFHA ILRQEIGWFD INDTTELNTR LTDDISKISEG I	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 196 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 40.550041 # Exp number, first 60 AAs 0.000000 # Total prob of N-in 0.647462 inside 1 62 TMhelix 63 86 outside 87 130 TMhelix 131 151 inside 152 196 	**NO**	**NO**	**NO**

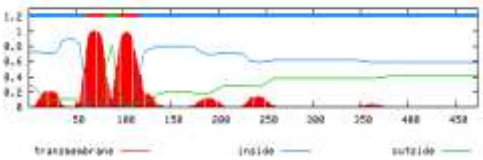
- The polypeptide sequence MDR3 AA (1-188) was selected for further narrowing down to find out most appropriate polypeptide segment that did not contain any post-translational modifications and also did not contain any restriction site for the restriction enzyme Eam1105I. This polypeptide segment of MDR3 protein fulfilled the necessary criteria to be used for creating fusion protein.

Table 4(b): Human P-Glycoprotein (AA, 140-296)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (140-296) LAAGRQIRKI RQKFFHAILR QEIGWFDIN DTTELNTRLT DDISKISEGIG DKVGMFFQ AVATFFAGFI VGFIRGWKL TLVIMAISPIL GLSAAVWA KILSAFSDKEL AAYAKAGAV AEEALGAIRT VIAFGGQNK ELERYQKHLE NAKEIGIKKA IS	#Name MDR3_HUMAN # Annotation TM PROTEIN # Length 157 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 38.441605 # Exp number, first 60 AAs 6.357216 # Total prob of N-in 0.313478 inside 1 52 TMhelix 53 72 outside 73 78 TMhelix 79 99 inside 100 157  	NO	NO	YES

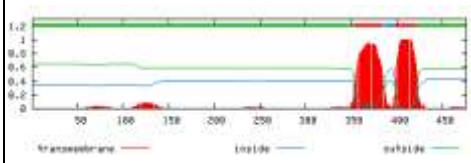
- The polypeptide sequence MDR3 AA (140-296) contained GPI-anchor signal in its sequence and hence excluded. Furthermore, information also shows that the probability of N-terminal end to be on the cytoplasmic side is lower (0.313) than 1. The N-terminal end could be a signal peptide as the value of expected first 60 AAs is more than a few (6.35). These informations were sufficient for excluding this polypeptide sequence.

Table 4(c): Human P-Glycoprotein (AA, 239-711)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (239-711) SAFSDKELAAYAKA GAVAEEALGAIRTVI AFGGQNKELERYQK HLENAKEIGIKKAISA NISMGIAFLLIYASYA LAFWYGSTLVISKEY TIGNAMTVFFSILIG AFSVGQAAPCIDAF ANARGAAYVIFDIID NNPKIDSFSERGHKP DSIKGNLEFNDVHFS YPSRANVKILKGLNL KVQSGQTVALVGSS GCGKSTTVQLIQRLY DPDEGTINIDGQDIR NFNVNYLREIIGVVS QEPVLFSTTIAENICY GRGNVTMDEIKKAV KEANAYEFIMKLPQ KFDTLVGERGAQLS GGQKQRIAIARALV RNPKILLLDEATSAL DTESEAEVQAALDK AREGRTTIVIAHRLS TVRNADVIAGFEDG VIVEQGSHSELMKK EGVYFKLVNMQTSG SQIQSEEFELNDEKA ATRMAPNGWKSRL FRHSTQKNLKNSQ MCQKSLDVETDGLE ANVPPVSFLKVLKLN KTEWPYF	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 473 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 37.343231 # Exp number, first 60 AAs 0.000000 # Total prob of N-in 0.725418 inside 1 60 TMhelix 61 81 outside 82 95 TMhelix 96 116 inside 117 473 	NO	YES	YES

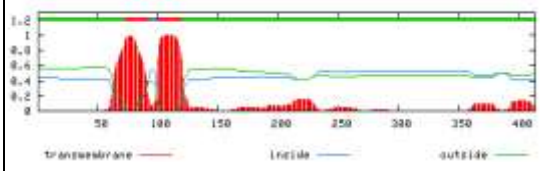
- The polypeptide sequence MDR3 AA (239-711) was excluded as it contained palmitoylation as well as GPI-anchor signal in its sequence. Though the membrane topology is good, this polypeptide segment couldn't be used.

Table 4(d): Human P-Glycoprotein (AA, 355-831)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (355-831) AFANARGAAYVIFDIID NNPKIDSFSERGHKPDSI KGNLEFNDVHFSYPSRA NVKILKGLNLKVQSGQT VALVGSSGCGKSTTVQL IQRLYDPDEGTINIDGQ DIRNFNVNYLREIIGVVS QEPVLFSTTIAENICYGR GNVTMDEIKKAVKEAN AYEFIMKLPQKFDTLVG ERGAQLSGGQKQRIAIA RALVRNPKILLLDEATSA LDTESEAEVQAALDKAR EGRTTIVIAHRLSTVRNA DVIAGFEDGVIVEQGSH SELMKKEGVYFKLVNM QTSGSQIQSEEFELNDE KAATRMAPNGWKSRLF RHSTQKNLKNSQMCQK SLDVETDGLEANVPPVS FLKVLKLNKTEWPYFVV GTVCAIANGGLQPAFSV IFSEIIAIFGPGDDAVKQ QKCNIFSLIFLFLGIISFFT FFLQGFTFGKAGEILTRR LRSMAFKAMLRQDMS WFDDHKNSTGALSTRL ATDAAQVQGATGTR	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 476 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 47.643551 # Exp number, first 60 AAs 0.000000 # Total prob of N-in 0.365605 outside 1 352 TMhelix 353 385 inside 386 397 TMhelix 398 421 outside 422 476 	NO	YES	YES

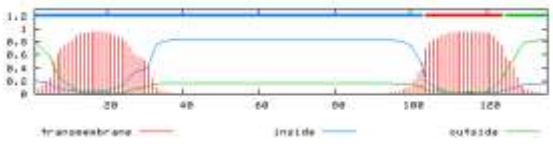
- The polypeptide MDR3 AA (355-831) was also excluded as it contained palmitoylation as well as GPI-anchor signal in its sequence. The membrane topology is in opposite orientation i.e., both the N- and C-terminal were outside the cell membrane.

Table 4(e): Human P-Glycoprotein (AA, 874-1286)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (874-1286) EMKLLAGNAKRD KKELEAAGKIATE AIENIRTVVSLTQE RKFESMYVEKLYG PYRNSVQKAHIYG ITFSISQAFMYFSY AGCFRFGAYLIVN GHMRFRDVILVFS AIVFGAVALGHAS SFAPDYAKAKLSA AHLFMLFERQPLI DSYSEEGLKPDKF EGNITFNEVVFNY PTRANVPVLQGL SLEVKKGQTLALV GSSGCGKSTVVQ LLERFYDPLAGTV FVDFGFQLLDGQ EAKKLNVQWLRA QLGIVSQEPILFDC SIAENIAYGDNSR VVSQDEIVSAAKA ANIHPFIETLPHKY ETRVGDKGTQLS GGQKQRIAIARAL IRQPQILLLDEATS ALDTESEKVVQEA LDKAREGRTCIVIA HRLSTIQNADLIV VFQNGRVKEHGT HQQLLAQKGIYFS MVSVQAGTQNL	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 413 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 35.467701 # Exp number, first 60 AAs 0.000000 # Total prob of N-in 0.424186 outside 1 72 TMhelix 73 93 inside 94 99 TMhelix 100 120 outside 121 413 	NO	NO	YES

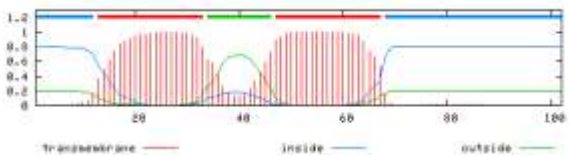
- The polypeptide MDR3 AA (874-1286) was also not used further as it contained GPI-anchor signal in its sequence. Its membrane topology is also in opposite orientation i.e., both the N- and C-terminal were outside the cell membrane

Table 5(a): Claudin Like Protein 24 (AA, 1-136)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (1-136) MTVQRLVAAA VLVALVSLILNN VAAFTSNWVC QTLEDGRRRSV GLWRSCWLVD RTRGGPSPGAR AGQVDAHDCE ALGWGSEAAG FQESRGTVKLQ FDMMRACNLV ATAALTAGQLT FLLGLVGLPLLS PDAPCWEE	# Name CLP24_HUMAN # Annotation TM PROTEIN # Length 136 # Number of predicted TMHs 1 # Exp number of AAs in TMHs 18.696194 # Exp number, first 60 AAs 0.000000 # Total prob of N-in 0.202570 inside 1 103 TMhelix 104 124 outside 125 136 	NO	YES	NO

- The polypeptide CLP24 AA (1-136) was excluded because the polypeptide segment was predicted to have only one transmembrane domain and also contained palmitoylation site in its amino acid sequence.

Table 5(b): Claudin Like Protein 24 (AA, 125-226)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
CLP24_ HUMAN	Amino acids: (125-226) PLLSPDAPCWE EAMAAAFQLA SFVLVIGLVTFY RIGPYTNLSWS CYLNIGACLLAT LAAAMLIWNIL HKREDCMAPR VIVISRSLTARF RRGLDNDYVES PC	# Name CLP24_HUMAN # Annotation TM PROTEIN # Length 102 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 37.794697 # Exp number, first 60 AAs 31.076910 # Total prob of N-in 0.796483 inside 1 12 TMhelix 13 33 outside 34 46 TMhelix 47 67 inside 68 102 	NO	YES	NO

- The polypeptide CLP24 AA (125-226) was selected for further narrowing down to find out more specific polypeptide segment that could be used in generating fusion protein of hSNAP-23cys^{-}. Although it contained palmitoylation site, it fulfilled other necessary criteria to be used for further narrowing down. We had to choose this polypeptide segment over the segment CLP24 AA (1-136), because the later contained only one transmembrane domain in addition to palmitoylation site in it.

According to the findings in above sections, some of transmembrane domains (AA 1-188 from MDR3 and AA 125-226 from CLP24) were finally narrowed down to three transmembrane domains that had desirable transmembrane domain that lacked post translational modifications.

- **Legends for tables 6 and 7:**

These polypeptide segments were narrowed down from the above selected polypeptide segments of MDR3 and CLP24 proteins that were found to have no post-translational modification and have correct orientation (membrane topology). Table 6(a), (b) and 7 shows the membrane topology along with their cartoon structure for final three polypeptide sequences that could be used for generating fusion proteins of t-SNARE SNAP-23. These polypeptide segments fulfils all the necessary criteria that were required for generating fusion proteins of t-SNARE SNAP-23 that do not goes to lipid raft region and could be used for studying the possible role of post translational modification of SNAP-23 in membrane association and membrane fusion.

Table 6(a): Human P-Glycoprotein (AA, 1-145)

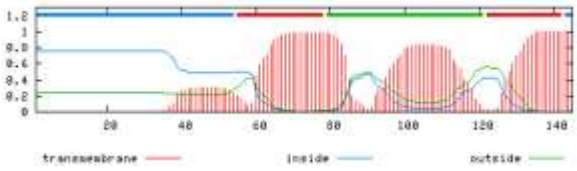
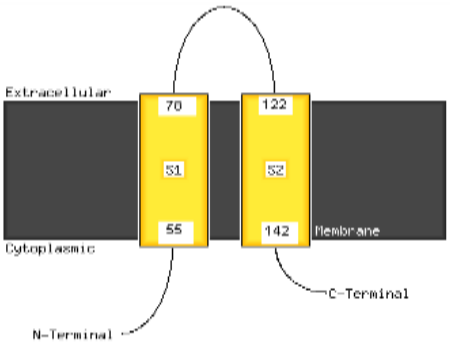
Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (1-145) MDLEAAKNGT AWRPTSAEGD FELGISSKQKRK KTKTVKMIGVL TLFRYSDWQD KLFMSLGTIMA IAHGSGLPLM MIVFGEMTDK FVDTAGNFSFP VNFSLSLLNPG KILEEEMTRYAY YYSGLGAGVLV AAYIQVSFWTL AAGRQ	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 145 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 40.212391 # Exp number, first 60 AAs 3.660151 # Total prob of N-in 0.727572 inside 1 54 TMhelix 55 78 outside 79 121 TMhelix 122 142 inside 143 145 a) Graphical representation  b) Cartoon structure of membrane topology  	NO	NO	NO

Table 6(b): Human P-Glycoprotein (AA, 41-145)

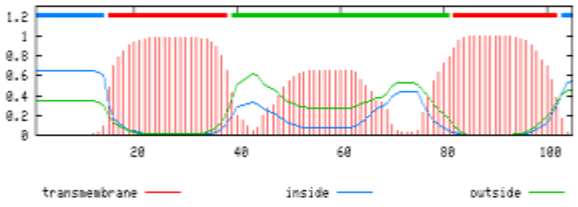
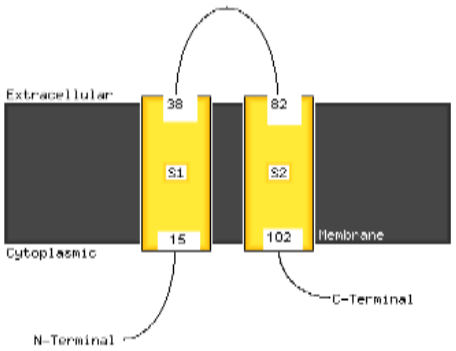
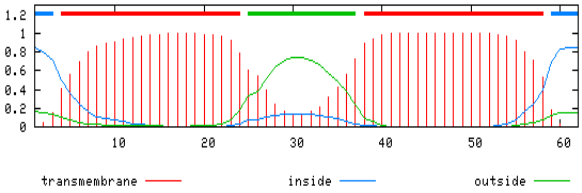
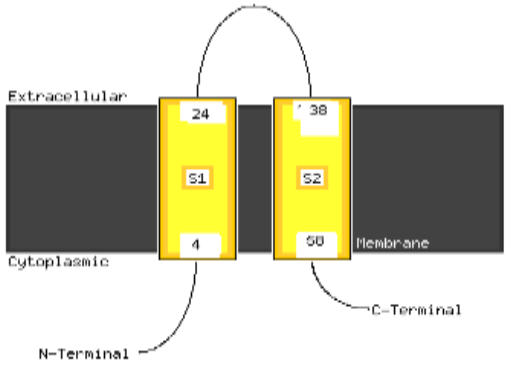
Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
MDR3_ HUMAN	Amino acids: (41-145) GVLTLFRYSDW QDKLFMSLGTI MAIAHGSGLPL MMIVFGEMTD KFVDTAGNFSF PVNFSLSLLNP GKILEEEMTRY AYYYSGLGAGV LVAAYIQVSFW TLAAGRQ	# Name MDR3_HUMAN # Annotation TM PROTEIN # Length 105 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 40.373383 # Exp number, first 60 AAs 21.571674 # Total prob of N-in 0.652431 inside 1 14 TMhelix 15 38 outside 39 81 TMhelix 82 102 inside 103 105 a) Graphical representation  b) Cartoon structure of membrane topology  	NO	NO	NO

Table 7: Claudin Like Protein 24 (AA, 134-195)

Protein Name	Polypeptide Sequence	TMMOD Data	Post Translational Modification		
			MYR	PALM	GPI
CLP24_ HUMAN	Amino acids: (134-195) WEEAMAAA FQLASFVLVI GLVTFYRIGP YTNLSWSCY LNIGACLLAT LAAAMLIWN ILHKRE	# Name CLP24_HUMAN # Annotation TM PROTEIN # Length 62 # Number of predicted TMHs 2 # Exp number of AAs in TMHs 38.267117 # Exp number, first 60 AAs 38.267117 # Total prob of N-in 0.839161 inside 1 3 TMhelix 4 24 outside 25 37 TMhelix 38 58 inside 59 62 a) Graphical representation  b) Cartoon structure of membrane topology 	NO	NO	NO

Third section as per the part of bioinformatics approach was to study the **expression** of each desired protein. Gene Expression analysis was performed by using the **'Gene X' (GeneCards) tool.**

4.3 Expression Profile in Various Human Tissues (GeneNote)

Duplicate measurements were obtained for twelve normal human tissues (out of 28 tissues shown) hybridized against Affymetrix GeneChips HG-U95A-E (GeneNote data) and for 22 normal human tissues hybridized against HG-U133A (GNF data). The intensity values (shown on the y-axis) were first averaged between duplicates, then probe set values were averaged per gene, global median-normalized and scaled to have the same median of about 70 (half-way between GeneNote and GNF medians). Normalized intensities are drawn on a root scale, which is an intermediate between log and linear scales.

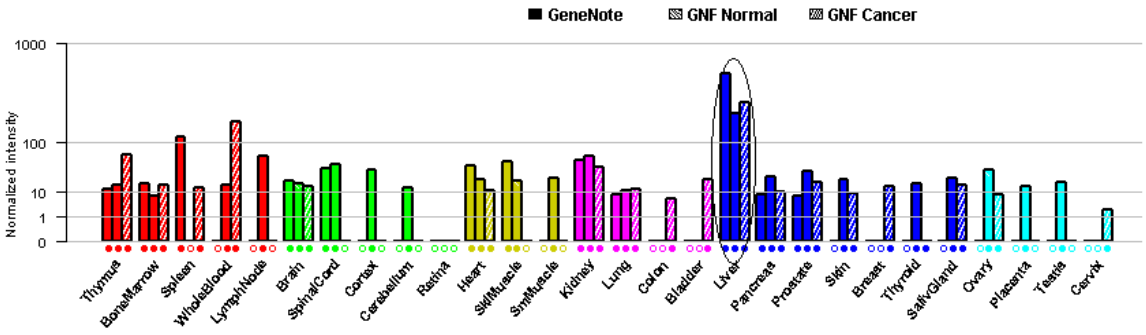


Figure 13(a): GeneNote / GNF Normal / GNF Cancer expression array image of MDR3

- **Legend:**

  Figure 13(a) shows gene expression profile of MDR3 gene in different healthy tissues as well as cancerous cell lines from human. Gene expression profile shows that human MDR3 gene is expressed at higher level in hepatic tissues (liver). HepG2 cell line is a hepatic carcinoma cell line that expresses MDR3 gene at higher level and could be used for isolating RNA for amplifying the desirable gene sequence by RT-PCR and then PCR.

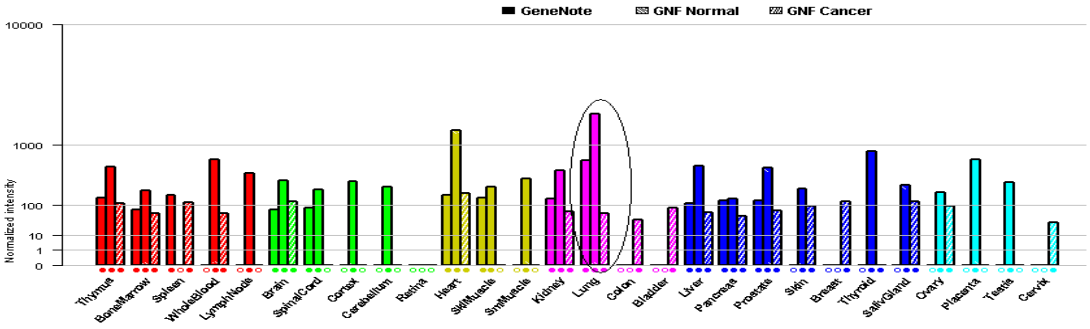


Figure 13(b): GeneNote / GNF Normal / GNF Cancer expression array image of CLP24

- **Legend:**

Figure 13(b) shows gene expression profile of CLP24 gene in different healthy tissues as well as cancerous cell lines from human. Gene expression profile shows that human CLP24 gene is expressed at higher level in Lungs. A549 cell line is a human alveolar basal epithelial carcinoma cell line that expresses CLP24 gene at higher level and was found to be a potential candidate for isolating RNA for amplifying the desirable gene sequence by RT-PCR and then PCR.

As per objective, the nucleotide fragment for the selected protein segment was cloned into hSNAP-23-cyc$^-$ at the unique restriction site in the linker in-silico and then the resulted recombinant protein was again checked for membrane topology and post-translational modification prediction using TMMOD online server and other online tools. Here is the result for the nucleotide and polypeptide sequence of the generated fusion protein and its predicted membrane topology.

```
1     ATGGATAATC TGTCATCAGA AGAAATTCAA CAGAGAGCTC ACCAGATTAC TGATGAGTCT
61    CTGGAAAGTA CGAGGAGAAT CCTGGGTTTA GCCATTGAGT CTCAGGATGC AGGAATCAAG
121   ACCATCACTA TGCTGGATGA ACAAAAGGAA CAACTAAACC GCATAGAAGA AGGCTTGGAC
181   CAAATAAATA AGGACATGAG AGAGACAGAG AAGACTTTAA CAGAACTCAA CAAATTCGCT
241   GGCCTTAATA GAACAAAGAA CTTTGAGTCT GGCAAGGCTT ATAAGACAAC ATGGGGAGAT
301   GGTGGAGAAA ACTCACCTTG CAATGTAGTA TCTAAACAGC CAGGCCCGGT GACAAATGGT
361   CAGATGGATC TTGAGGCGGC AAAGAACGGA ACAGCCTGGC GCCCCACGAG CGCGGAGGGC
421   GACTTTGAAC TGGGCATCAG CAGCAAACAA AAAAGGAAAA AAACGAAGAC AGTGAAAATG
481   ATTGGAGTAT TAACATTGTT TCGATACTCC GATTGGCAGG ATAAATTGTT TATGTCGCTG
541   GGTACCATCA TGGCCATAGC TCACGGATCA GGTCTCCCCC TCATGATGAT AGTATTTGGA
601   GAGATGACTG ACAAATTTGT TGATACTGCA GGAAACTTCT CCTTTCCAGT GAACTTTTCC
661   TTGTCGCTGC TAAATCCAGG CAAAATTCTG GAAGAAGAAA TGACTAGATA TGCATATTAC
721   TACTCAGGAT TGGGTGCTGG AGTTCTTGTT GCTGCCTATA TACAAGTTTC ATTTTGGACT
781   TTGGCAGCTG GTCGACAGAC AAATGGTCAG CTTCAGCAAC CAACAACAGG AGCAGTCAGT
841   GGTGGATACA TTAAACGCAT AACTAATGAT GCCAGAGAAG ATGAAATGGA AGAGAACCTG
901   ACTCAAGTGG GCAGTATCCT GGGAAATCTA AAAGACATGG CCCTGAACAT AGGCAATGAG
961   ATTGATGCTC AAAATCCACA AATAAAACGA ATCACAGACA AGGCTGACAC CAACAGAGAT
1021  CGTATTGATA TTGCCAATGC CAGAGCAAAG AAACTCATTG ACAGCTAA
```

Figure 14(a): Nucleotide Sequence of fusion protein hSNAP-23cys$^-$-MDR3, 1-145

```
MDNLSSEEIQQRAHQITDESLESTRRILGLAIESQDAGIKTITMLDEQKEQLNRIEEGLDQINKDMRETEKTLTELNKFAGLN
RTKNFESGKAYKTTWGDGGENSPCNVVSKQPGPVTNGQMDLEAAKNGTAWRPTSAEGDFELGISSKQKRKKTKTVKMI
GVLTLFRYSDWQDKLFMSLGTIMAIAHGSGLPLMMIVFGEMTDKFVDTAGNFSFPVNFSLSLLNPGKILEEEMTRYAYYY
SGLGAGVLVAAYIQVSFWTLAAGRQTNGQLQQPTTGAVSGGYIKRITNDAREDEMEENLTQVGSILGNLKDMALNIGNE
IDAQNPQIKRITDKADTNRDRIDIANARAKKLIDS
```

Figure 14(b): Amino acid sequence of fusion protein hSNAP-23cys$^-$-MDR3, 1-145

# Name	hSNAP-23-MDR3 (1-145)	
# Annotation	NON-TM PROTEIN	
# Length	355	
# Number of predicted TMHs	2	
# Exp number of AAs in TMHs	39.952724	
# Exp number, first 60 AAs	0.000000	
# Total prob of N-in	0.678287	
inside	1	175
TMhelix	176	199
outside	200	242
TMhelix	243	263
inside	264	355

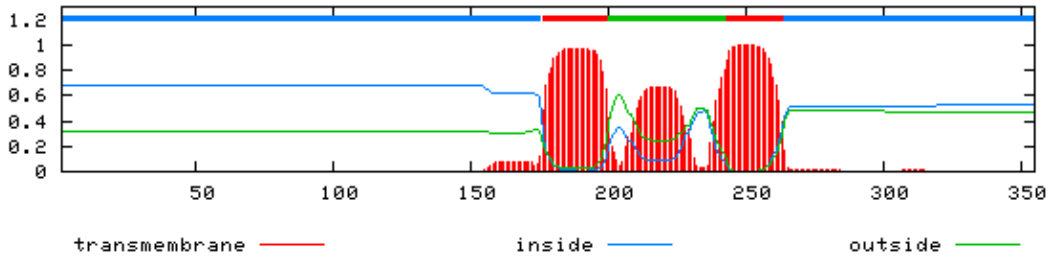


Figure 14(c): Predicted Membrane topology of fusion protein hSNAP-23cys$^-$-MDR3, 1-145

➢ **Legend:**

Figure 14(a) and 14(b) shows the nucleotide sequence and polypeptide sequence respectively for fusion protein hSNAP-23cys$^-$-MDR3, 1-145. Figure 14(c) shows the membrane topology of the fusion protein which is predicted by TMMOD server. This fusion protein contains two transmembrane domains which is present in the linker region of the hSNAP-23cyc$^-$ protein. The fusion protein also does not show any post-translational modifications.

```
1     ATGGATAATC TGTCATCAGA AGAAATTCAA CAGAGAGCTC ACCAGATTAC TGATGAGTCT
61    CTGGAAAGTA CGAGGAGAAT CCTGGGTTTA GCCATTGAGT CTCAGGATGC AGGAATCAAG
121   ACCATCACTA TGCTGGATGA ACAAAAGGAA CAACTAAACC GCATAGAAGA AGGCTTGGAC
181   CAAATAAATA AGGACATGAG AGAGACAGAG AAGACTTTAA CAGAACTCAA CAAATTCGCT
241   GGCCTTAATA GAACAAAGAA CTTTGAGTCT GGCAAGGCTT ATAAGACAAC ATGGGGAGAT
301   GGTGGAGAAA ACTCACCTTG CAATGTAGTA TCTAAACAGC CAGGCCCGGT GACAAATGGT
361   CAGGGAGTAT TAACATTGTT TCGATACTCC GATTGGCAGG ATAAATTGTT TATGTCGCTG
421   GGTACCATCA TGGCCATAGC TCACGGATCA GGTCTCCCCC TCATGATGAT AGTATTTGGA
481   GAGATGACTG ACAAATTTGT TGATACTGCA GGAAACTTCT CCTTTCCAGT GAACTTTTCC
541   TTGTCGCTGC TAAATCCAGG CAAAATTCTG GAAGAAGAAA TGACTAGATA TGCATATTAC
601   TACTCAGGAT TGGGTGCTGG AGTTCTTGTT GCTGCCTATA TACAAGTTTC ATTTTGGACT
661   TTGGCAGCTG GTCGACAGAC AAATGGTCAG CTTCAGCAAC CAACAACAGG AGCAGTCAGT
721   GGTGGATACA TTAAACGCAT AACTAATGAT GCCAGAGAAG ATGAAATGGA AGAGAACCTG
781   ACTCAAGTGG GCAGTATCCT GGGAAATCTA AAAGACATGG CCCTGAACAT AGGCAATGAG
841   ATTGATGCTC AAAATCCACA AATAAAACGA ATCACAGACA AGGCTGACAC CAACAGAGAT
901   CGTATTGATA TTGCCAATGC CAGAGCAAAG AAACTCATTG ACAGCTAA
```

Figure 15(a): Nucleotide Sequence of fusion protein hSNAP-23cys^{-}-MDR3, 41-145

```
MDNLSSEEIQQRAHQITDESLESTRRILGLAIESQDAGIKTITMLDEQKEQLNRIEEGLDQINKDMRETEKTLTELNKFAGLN
RTKNFESGKAYKTTWGDGGENSPCNVVSKQPGPVTNGQGVLTLFRYSDWQDKLFMSLGTIMAIAHGSGLPLMMIVFGE
MTDKFVDTAGNFSFPVNFSLSLLNPGKILEEEMTRYAYYYSGLGAGVLVAAYIQVSFWTLAAGRQTNGQLQQPTTGAVS
GGYIKRITNDAREDEMEENLTQVGSILGNLKDMALNIGNEIDAQNPQIKRITDKADTNRDRIDIANARAKKLIDS
```

Figure 15(b): Amino acid sequence fusion protein of hSNAP-23cys^{-}-MDR3, 41-145

#Name	hSNAP-23-MDR3 (41-145)	
# Annotation	NON-TM PROTEIN	
# Length	315	
# Number of predicted TMHs	2	
# Exp number of AAs in TMHs	40.123978	
# Exp number, first 60 AAs	0.000000	
# Total prob of N-in	0.699449	
inside	1	135
TMhelix	136	159
outside	160	202
TMhelix	203	223
inside	224	315

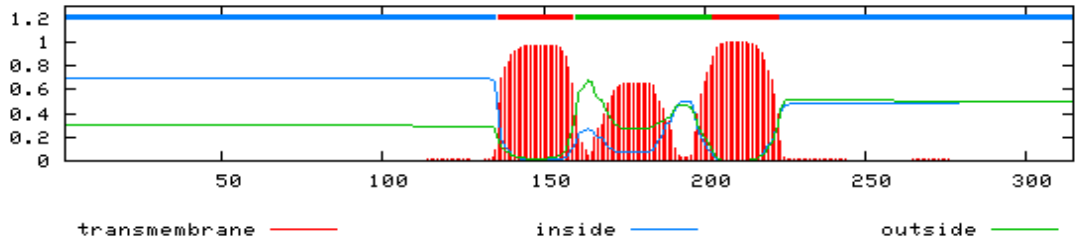


Figure 15(c): Predicted Membrane topology of fusion protein hSNAP-23cys$^-$-MDR3, 41-145

➢ **Legend:**

Figure 15(a) and 15(b) shows the nucleotide sequence and polypeptide sequence respectively for fusion protein hSNAP-23cys$^-$-MDR3, 41-145. Figure 15(c) shows the membrane topology of the fusion protein which is predicted by TMMOD server. This fusion protein contains two transmembrane domains which is present in the linker region of the hSNAP-23cyc$^-$ protein. The fusion protein also does not show any post-translational modifications.

```
1     ATGGATAATC TGTCATCAGA AGAAATTCAA CAGAGAGCTC ACCAGATTAC TGATGAGTCT
61    CTGGAAAGTA CGAGGAGAAT CCTGGGTTTA GCCATTGAGT CTCAGGATGC AGGAATCAAG
121   ACCATCACTA TGCTGGATGA ACAAAAGGAA CAACTAAACC GCATAGAAGA AGGCTTGGAC
181   CAAATAAATA AGGACATGAG AGAGACAGAG AAGACTTTAA CAGAACTCAA CAAATTCGCT
241   GGCCTTAATA GAACAAAGAA CTTTGAGTCT GGCAAGGCTT ATAAGACAAC ATGGGGAGAT
301   GGTGGAGAAA ACTCACCTTG CAATGTAGTA TCTAAACAGC CAGGCCCGGT GACAAATGGT
361   CAGTGGGAGG AGGCCATGGC CGCTGCATTC CAACTGGCGA GTTTTGTCCT GGTCATCGGG
421   CTCGTGACTT TCTACAGAAT TGGCCCATAC ACCAACCTGT CCTGGTCCTG CTACCTGAAC
481   ATTGGCGCCT GCCTTCTGGC CACGCTGGCG GCAGCCATGC TCATCTGGAA CATTCTCCAC
541   AAGAGGGAGA CAAATGGTCA GCTTCAGCAA CCAACAACAG GAGCAGTCAG TGGTGGATAC
601   ATTAAACGCA TAACTAATGA TGCCAGAGAA GATGAAATGG AAGAGAACCT GACTCAAGTG
661   GGCAGTATCC TGGGAAATCT AAAAGACATG GCCCTGAACA TAGGCAATGA GATTGATGCT
721   CAAAATCCAC AAATAAAACG AATCACAGAC AAGGCTGACA CCAACAGAGA TCGTATTGAT
781   ATTGCCAATG CCAGAGCAAA GAAACTCATT GACAGCTAA
```

Figure 16(a): Nucleotide Sequence of fusion protein hSNAP-23cys^{-}-CLP24, 134-195

```
MDNLSSEEIQQRAHQITDESLESTRRILGLAIESQDAGIKTITMLDEQKEQLNRIEEGLDQINKDMRETEKTLTELNKFAGLN
RTKNFESGKAYKTTWGDGGENSPCNVVSKQPGPVTNGQWEEAMAAAFQLASFVLVIGLVTFYRIGPYTNLSWSCYLNIG
ACLLATLAAAMLIWNILHKRETNGQLQQPTTGAVSGGYIKRITNDAREDEMEENLTQVGSILGNLKDMALNIGNEIDAQN
PQIKRITDKADTNRDRIDIANARAKKLIDS
```

Figure 16(b): Amino acid sequence of fusion protein hSNAP-23cys^{-}-CLP24, 134-195

#Name	hSNAP-23–CLP24 (134-195)	
# Annotation	TM PROTEIN	
# Length	272	
# Number of predicted TMHs	2	
# Exp number of AAs in TMHs	38.277306	
# Exp number, first 60 AAs	0.000000	
# Total prob of N-in	0.783624	
inside	1	126
TMhelix	127	147
outside	148	158
TMhelix	159	179
inside	180	272

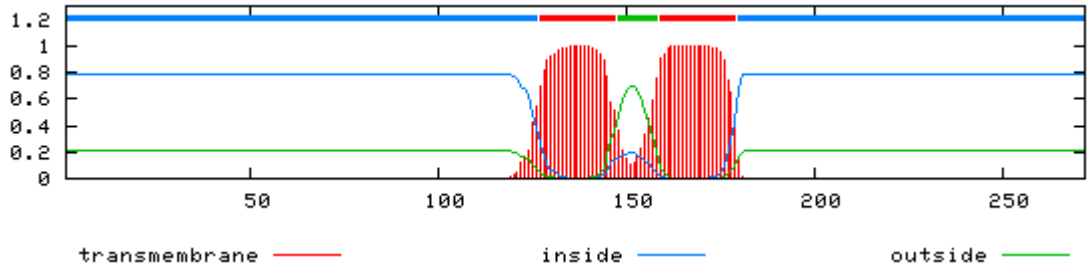


Figure 16(c): Predicted Membrane topology of fusion protein hSNAP-23cys⁻-CLP24, 134-195

➢ **Legend:**

Figure 16(a) and 16(b) shows the nucleotide sequence and polypeptide sequence respectively for fusion protein hSNAP-23cys⁻-MDR3, 41-145. Figure 16(c) shows the membrane topology of the fusion protein which is predicted by TMMOD server. This fusion protein contains two transmembrane domains which is present in the linker region of the hSNAP-23cyc⁻ protein. The fusion protein also does not show any post-translational modifications

- **Key for figure 14, 15 and 16**

ACGT – hSNAP-23

ACGT – hSNAP-23 Linker Region

ACGT – Cloned Gene

ACGT – Extra AA (due to cloning and ligation)

4.4 Generation of Fusion Protein

4.4.1 Amplification of Plasmid DNA

4.4.1.1 Transformation

Table 8: Determination of transformation efficiency of plasmid DNA

S. No.	Plasmid used	Amount of plasmid DNA added (µg)	No. of colonies observed	Transformation efficiency
1	pEGFPN2	60×10^{-4}	178	2.96×10^{3}
2	pEGFPS23-Cys-	36×10^{-4}	142	3.94×10^{3}

Table 9: Determination of amount of DNA and purity by Nanodrop ND3000 Spectrophotometer

S.No.	Name of the plasmid	Amount of DNA (µg)	Purity ratio ($A_{260}/A_{280} \geq 1.8$)
1	pEGFP	1750	1.91
2	pEGFPS23-Cys^{-}	132.5	1.87

4.4.1.2 Checking Plasmid DNA by Restriction Digestion

In order to confirm that the right plasmid DNA had been amplified by maxipreparation, the plasmid samples were linearized by restriction digestion and then run on gel. The restriction digestion of the plasmid pEGFP and pEGFPS23-Cys$^-$ showed (Figure 17) that the size of the wild type plasmid (pEGFP) is 4.7 kbp and that of Cysteine mutant plasmid (pEGFPS23-Cys$^-$) is 5.5 kbp. The digested plasmid is linear and hence showed single band as compared to native undigested plasmid which shows multiple bands.

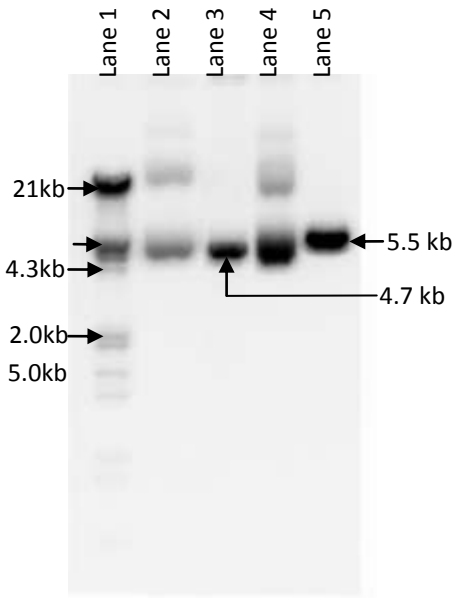


Figure 17: BamHI digested Transformed Plasmid DNA

Lane 1: λ DNA (EcoRI+HindIII digested) marker (from Biorad)
Lane 2: Undigested DNA sample of pEGFP
Lane 3: Digested DNA sample of pEGFP (4.7 kbp)
Lane 4: Undigested DNA sample of pEGFPS23-Cys$^-$
Lane 5: Digested DNA sample of pEGFPS23-Cys$^-$ (5.5 kbp)

4.4.2 Maintaining HeLa Cell Culture

HeLa cells were observed as adherent cell lines sticking to the wall of the flask, growing in monolayer, polygonal in shapes with prominent nucleus. The cells were confluent and in healthy looking condition as shown in the figure 18. This figure shows the in-vitro cultured HeLa cells in RPMI-CM.

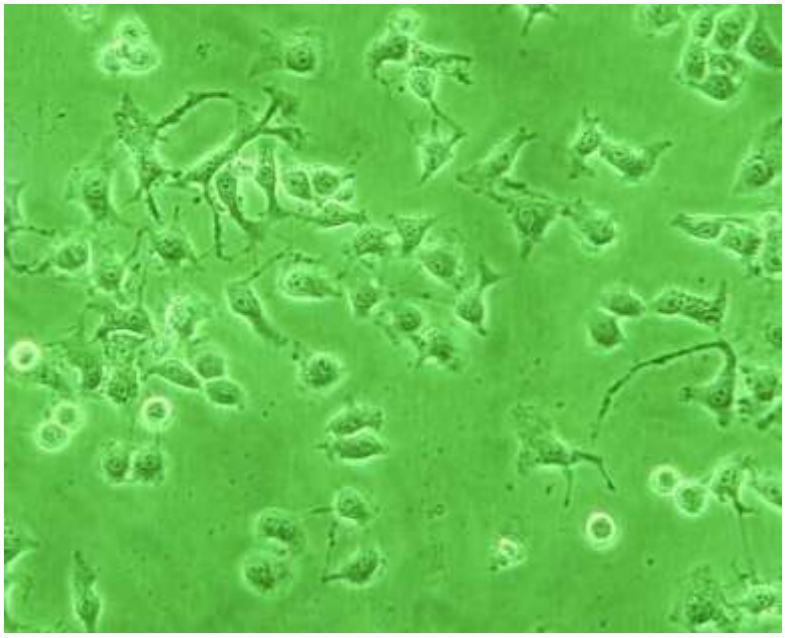


Figure 18: HeLa cells in culture (40X, Nikon Eclipse TS100)

4.4.2.1 Percentage Viability of HeLa Cell Culture

Percentage viability showed that the cells cultured in-vitro were growing well and were showing almost 100% viability up to 12 hrs post culture after which the viability decreased gradually. Table 10 shows the percentage viability of HeLa cells in culture which is represented graphically in figure 19.

Table 10: Percentage Viability of HeLa Cells

S.No.	Time	Live Cells/ml (average)	Dead Cells/ml (average)	% Viability
1	0 hr	20,000	-	100 %
2	6 hrs	21,000	-	100 %
3	12 hrs	24,000	200	99.17 %
4	24 hrs	26,500	1,500	94.64 %
5	36 hrs	31,000	5,000	86.11 %

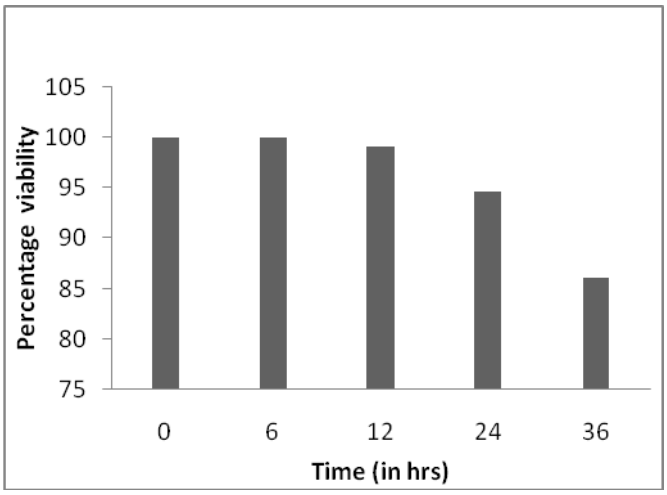


Figure 19: Graphical representation of Percentage viability of HeLa cell culture

4.4.3 Isolation of Total RNA from HeLa and A549 Cells

RNA was isolated from HeLa cell lines and A549 cell lines (in duplicate) by Trizol reagent method. RNA recoveries were estimated by using Nanodrop ND3000 Spectrophotometer. The Nanodrop readings in table 11 shows that the isolated RNA was of good quality and satisfactory concentration. Amount of RNA isolated was higher from HeLa cells as compared to A549 cells. Table 12 shows the comparison of RNA yield from two different cell lines. The RNA recovery was gradually increasing with the increase in cell number (as shown in figure 20). This indicates that the RNA recovery is directly proportional to cell number keeping other parameters constant.

Table 11: Nanodrop readings for different RNA samples

Sample	**Number of cells used**	**Amount of Trizol used**	**$A_{260/280}$**	**$A_{260/230}$**	**Conc. (µg/µl)**	**Total RNA yield (in µg)**	**Average** (µg/million)
HeLa cells	$10x10^6$	1 ml	1.67	2.09	2.69	80.7	8.13
	$10x10^6$	1ml	1.69	2.26	2.73	81.9	
A549 cells	$15x10^6$	1.5 ml	1.59	1.94	2.80	83.7	5.76
	$15x10^6$	1.5 ml	1.60	1.99	2.97	89.1	

Table 12: RNA recovery from HeLa cells

S.No.	Cell number (HeLa Cells)	RNA recovery (in µg)
1	3.0×10^6	40. 49
2	5.0×10^6	54.20
3	7.0×10^6	78.60
4	10.0×10^6	91.20

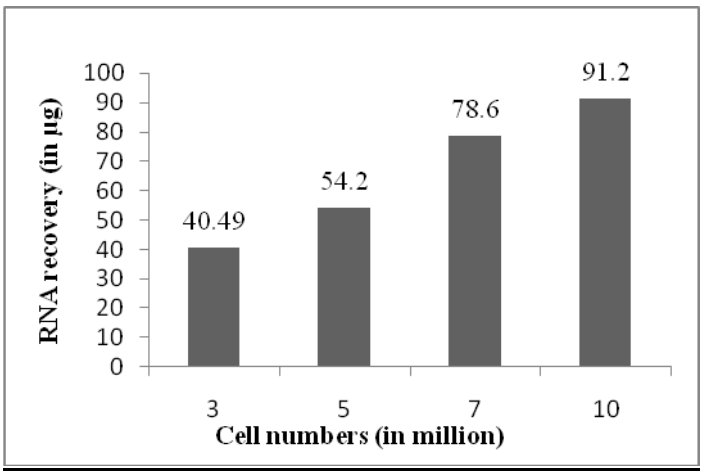
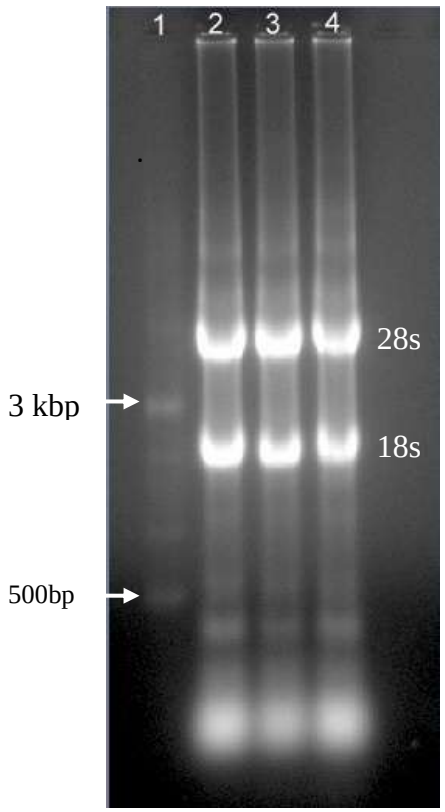


Figure 20: Graphical representation of RNA recovery from HeLa cells

4.4.4 Formaldehyde Agarose Gel Electrophoresis for RNA and cDNA

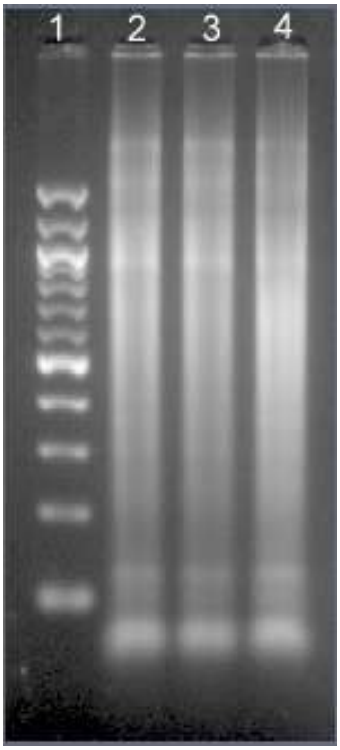
RNA gel showed two distinct bands of 28s and 18s for RNA samples along with 7 bands of ssRNA ladder (as shown in figure 21). Quality of the isolated RNA was good from both the cells lines. The TRIZOL reagent used for isolating RNA yielded good quality of RNA with satisfactory amount.

RT-PCR product was run onto 1.8 % agarose gel. The cDNA synthesized was of good quality as it showed 6 to 7 bands with smear on the gel. This cDNA was further used for PCR.



Lane 1: ssRNA ladder (500 to 9,000 bp) (from NEB)
Lane 2: HeLa RNA (3 µg)
Lane 3: HeLa RNA (3 µg)
Lane 4: A549 RNA (3 µg)

Figure 21: Quality of isolated RNA from different cells (1.2 % formaldehyde agarose gel)

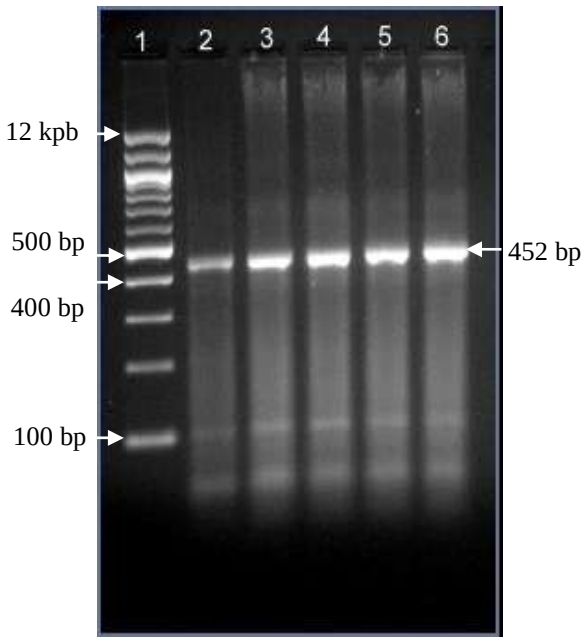


Lane 1: 100 bp DNA ladder (0.5 µg) (from NEB)
Lane 2: HeLa cDNA (3µl)
Lane 3: A549 cDNA (3µl)
Lane 4: A549 cDNA (3µl)

Figure 22: Quality of cDNA (RT PCR product) on 1.8 % agarose gel

4.4.5 Gradient PCR for Human GAPDH Gene

cDNA quality was also checked by doing gradient PCR for human GAPDH gene. Figure 14 shows the DNA band for the amplified hGAPDH gene which was synthesized by using the same cDNA (synthesized using HeLa RNA). The size of the amplified DNA product is 452 bp. All the annealing temperature that were used has worked with fine results at 52^{o}C and above.

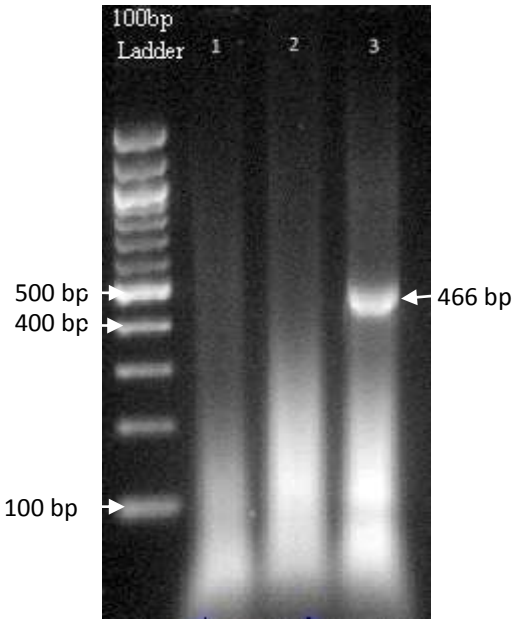


Lane 1: 100 bp DNA ladder (0.5 µg)
Lane 2: 50^{o}C
Lane 3: 51^{o}C
Lane 4: 52^{o}C
Lane 5: 53^{o}C
Lane 6: 54^{o}C
} Annealing Temperature

Figure 23: Gradient PCR for human GAPDH gene (positive control) from HeLa cDNA

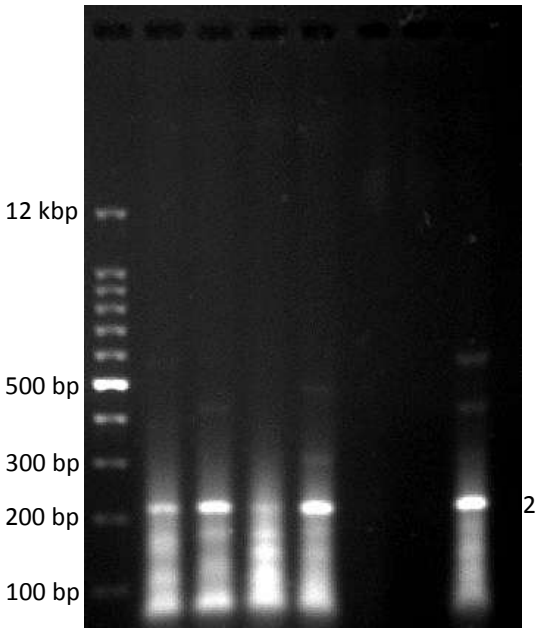
4.4.6 PCR for MDR3 (AA, 1-145) and CLP-24 (AA, 134-195) Using QIAGEN OneStep RT-PCR Kit

The amplified DNA fragment for MDR3 (AA, 1-145) was of 496 bp and was observed on the gel corresponding to the 500 bp DNA ladder band (shown in figure 24). Similarly, amplified DNA fragment of CLP-24 (AA, 134-195) were observed as distinct band on the agarose gel (shown in figure 25) whose size was 217 bp. PCR using normal Taq polymerase did not work for amplifying the gene segment using the gene specific primers because the primers contained non-complementary overhangs in them.



Lane 1 & 2: Negative control without template RNA
Lane 3: PCR product for MDR3 (AA, 1-145)

Figure 24: PCR product for MDR3 (AA, 1-145) using QIAGEN OneStep RT-PCR kit



Lane 1: 100 bp DNA ladder, (0.5 µg, from NEB)
Lane 2: 50.1°C
Lane 3: 53.2°C
Lane 4: 51.7°C
Lane 5: 52.4°C
Lane 8: 50.6°C
} annealing temperatures

217 bp (CLP24 PCR product)

Figure 25: PCR product for CLP24 (AA, 134-195) using QIAGEN OneStep RT-PCR kit

5. DISCUSSION

Associated with each membrane is a set of membrane proteins that enables the membrane to carry out its distinctive functions. The proteins that are attached to a membrane vary depending on cell type and sub-cellular location. Some proteins are bound only to the membrane surface, whereas others attach to membrane by certain hydrophobic handles like S-palmitoyl group or myristoyl chain added by post-translational modification of proteins. Domains lying along the cytosolic face of the membrane have a wide range of functions; from anchoring cytoskeletal proteins to the membrane to triggering intracellular signaling pathways. SNAREs are the class of membrane proteins that mediate membrane-membrane fusion in eukaryotic cells. SNAREs on target membranes are termed as t-SNAREs. While most of the SNAREs anchor in the membrane through a transmembrane (TM) domain, SNAREs belonging to the SNAP-23/SNAP-25 family do not possess a TM domain. Very recently it has been found that the membrane SNAP-23 anchors in membrane through S-palmitoylation. But, no studies have been done on importance of S-palmitoylation on function of SNAP-23, the ubiquitously expressed homologue of SNAP-25, which has been shown to have a major role in many immune cell effector functions. Previous studies have clearly shown that SNAP-23, phospho-SNAP-23 (generated in response to signaling for regulated exocytosis) as well as ternary SNARE complexes containing SNAP-23 are highly enriched in lipid rafts (Puri and Roche, 2006). Also SNAP-25 which does not associate with lipid rafts as efficiently as SNAP-23, does not support regulated exocytosis from mast cells to some extent. Recent studies also suggest that lipid rafts, cholesterol and sphingolipid-rich microdomains play an essential role in regulated exocytosis (Salaun and James, 2004).

The proteins were searched in different protein databases which included Interpro, UniprotKB/Swiss-Prot, ExPASy and NCBI protein database. Interpro is an integrated documentation resource for protein families, domains, regions and sites. Interpro combines a number of databases that use different methodologies and a varying degree of biological information on well-characterized proteins to derive protein signatures. By uniting the member databases, Interpro capitalizes on their individual strengths producing a powerful integrated database and diagnostic tool known as InterProScan (Quevillon *et al*., 2005). The literature mining was helpful in eliminating the proteins whose function was not clearly cited. The narrowing down of protein search was performed manually by studying about each protein individually. The topology prediction was performed with TMMOD: Hidden Markov Model for Transmembrane Protein Topology Prediction. The topology prediction helped to find out the membrane

topology of the individual polypeptide segments which further assisted in narrowing down of polypeptide segments to those that contained at least two transmembrane domains connected by an extracellular linker region and with both the free ends within the cytoplasm. The Polypeptide segments that contained single trans-membrane domain were eliminated. After that the selected polypeptide segments were analyzed for post-translational modification in them. The polypeptide segment that contained the post-translational modifications like palmitoylation, myristoylation and GPI-anchor site were eliminated because we were trying to relocate the SNAP-23 to non-raft region of the cytoplasmic leaflet of membrane. The hSNAP-23-cyc$^-$ has been found to prevent the membrane association of the protein SNAP-23 due to lack of palmitoylation in them.

Although most of the post-translational lipid modifications in protein are reversible, S-palmitoylation also called as thio-acylation or S-acylation could be reversible and attaches 16-carbon saturated fatty acid chain to specific cysteine residue in protein through thioester linkages (Bijlmakers and Marsh, 2003). Palmitoylation enhances the hydrophobicity and thus membrane affinity of proteins and helps in modulating protein trafficking (Draper *et al*., 2007) and sorting (Greaves and Chamberlain, 2007). Protein palmitoylation has also been found to mediate several cellular processes including signalling (Kurayoshi *et al*., 2007), apoptosis (Chakrabandhu *et al*., 2007) and neuronal transmission (Roth *et al*., 2006). CSS-Palm is a software that could predict out potential palmitoylation sites for ~1000 proteins (with an average length of ~1000aa) and used in this study for potential palmitoylation site prediction in the polypeptide sequences. The CSS-Palm 3.0 is freely available software tool (Ren *et al*., 2008). NMT-MYR was the tool that was used for predicting myristoylation site in polypeptide sequences. This software was available freely online.

Eukaryotic proteins that are associated with plasma membranes carry a Glycosylphosphatidylinositol (GPI) anchor. Membrane anchor through a GPI-anchor can serve as an apical localization signal in most cell types. This GPI anchors are linked to the C-terminal residue after a proteolytic cleavage occurring at a specific site known as ω-site (Pierleoni *et al*., 2008). All GPI-anchors have structural similarities, with minor differences among species to species. The core of the anchor molecule comprises a sugar moiety and a phosphatidylinositol molecule, linked to two long chain fatty acids. The sugar molecule can form amide bond with the C-terminal residue of a polypeptide. Free GPI-anchors are present normally to the plasma membrane where proteins are covalently bound after post-translational modifications occurring in the endoplasmic reticulum. Predictive methods are available online in order to recognize GPI-anchored proteins and to determine the ω-site within the whole protein sequence. Different methods that are available differ on the computational method adopted to develop the

algorithm. More recently GPI-SOM uses self-organizing maps (SOM), achieving a better performance than the previously described methods in discriminating GPI-anchored proteins (Fankhauser and Maser, 2005). Here we have used GPI-SOM for predicting the potential GPI-anchor signal in the polypeptide sequences.

GeneNote is a full-genome database of expression in healthy human tissues and contains raw data, normalized data and various analyses. The attainment of genomic expression data is based on high-density DNA microarray (Affymetrix GeneChip HG-U95A-E) consisting of 62,839 probe-sets representative of the full human genome. Tissue specific genes are supposed to have a significant role in tissue functionality. Tissue specificity of a particular gene is defined on the basis of as having a 'absent-present' calls of the Affymetrixx MAS 5.0 software. The MAS 5.0 package is one of the most widely used software tools for analyzing high-density microarray results (Shmueli *et al*., 2003). The interpretation of the RNA expression data requires linking between probe sequence and the gene they represent and acquiring broad biological knowledge regarding each gene's function, biological processes and counterparts. Via GeneCards projects, such information known as gene annotation is connected to the expression profiles. Gene annotation is essential for understanding the cellular program and the molecular physiology of an organism. GeneCards is a searchable, integrated database of human genes that provides brief genomic related information, on all known and predicted genes. We have used this tool for identifying the possible human tissues where expression of MDR3 and CLP24 genes were found to be higher.

The goal in protein engineering is to identify specific sequence changes that endow proteins with desired functional properties. As opposed to traditional rational and random protein engineering techniques, we have employed a bioinformatic approach to identify three specific transmembrane domains that could be inserted in the linker region of SNAP-23 to generate the fusion proteins. Fusion proteins are proteins created through the joining of two or more genes which originally code for separate proteins. Translation of this fusion gene results in a single polypeptide with functional properties derived from each of the original proteins.

Fusion proteins were constructed in-silico by inserting transmembrane domain from another protein from non-raft region. The protein was selected from human or rat by using protein databases (UniProt, InterPro, NCBI, ExPASy) and by using online analysis tools. As there was a need to extract the TM protein, expression analysis through GENECARDS was performed. The obtained fusion proteins were again subjected to the analysis by bioinformatic tools.

SNAP-23 are synthesized as soluble proteins and become membrane associated via palmitoylation of their respective cysteine-rich domains. SNAP-23 is a t-SNARE that is supposed to be associated to the plasma membrane by palmitoylation of its five cysteine molecules present in its linker region. Other types of SNARE proteins are embedded in the membrane by their hydrophobic peptide region. SNAP-23 is one of the major SNARE proteins that mediate SNARE complex formation during membrane fusion event. We can assume that if SNAP-23 is not present in the raft region of the membrane where SNARE complex is expected to buildup then membrane fusion event would be affected adversely. Membrane fusion is the most important event that regulates exocytosis and endocytosis pathways. Both of these processes are vital for the export and import of various molecules and components into and outside the cell that are required for proper cell functioning.

For isolating the specific transmembrane domains, firstly RNA was isolated from the cell lines in which expression level of the desirable gene was higher. The RNA was then reverse transcribed to cDNA which was then used for amplifying the specific gene segment using the gene specific primer. The amplified gene segment could be cloned into a suitable vector system containing SNAP-23cys^{-} gene using specific restriction enzyme. The vector could be then transfected into appropriate expression system for determining the expression of fusion protein and studying its localization in the cells.

Once the desirable gene has been inserted into the vector system containing SNAP-23, its expression could be studied in-vivo. The generated fusion proteins showed no post translational modification in-silico while checking with online bioinformatic tools. In this study, though the expression was not studied in the cell lines, it is expected that it would not go to raft region of the membrane. Bioinformatic studies shows that the fusion protein should not be targeted to raft region of the plasma membrane. Hence we can conclude that it might prevent exocytosis that is mediated by membrane fusion.

Formaldehyde agarose gel is used for visualizing RNA bands. Formaldehyde is used to denature the ribosomal RNA which can then be separated in two distinct bands of 28s and 18s which are components of the ribosomal RNA. The cDNA synthesized showed 7 to 8 faint bands. As the amplified cDNA is of variable sizes of different genes the bands were not so distinct. The Oligo (dT) primers were used in this experiment to synthesize the cDNa which is specific and complementary with the polyA tail of the mRNAs. Hence, all the RNA that contains polyA tail intact is supposed to be reversed transcribed into cDNA.

The gradient PCR for human GAPDH gene was performed in order to check the quality of RNA and cDNA synthesized. The result showed that the RNA was of good quality and could be used for the RT-PCR for amplification of the desirable gene. But, since the primers for MDR3 and CLP24 contained an extra overhang region of non-complementary nucleotides, normal Taq polymerase did not worked efficiently for the desirable genes to be amplified. Hence, we used QIAGEN OneStep PCR kit for amplifying the DNA fragments of MDR3 (AA, 1-145) and CLP24 (AA, 134-195). This kit uses RNA as template to directly amplify the desirable DNA fragment with the help of specific primers. Reverse transcriptase and PCR are carried out sequentially in the same tube. It has high specificity and sensitivity for amplifying the genes that are expressed at lower level. The PCR products for MDR3 (AA, 1-145) and CLP24 (AA, 134-195) were expected to be of 466 bp and 217 bp respectively which was confirmed from the agarose gel. The size of the DNA amplicons were slightly larger than compared to corresponding polypeptide sequence. This is because, the amplicon contained additional nucleotide sequence of the primers that is shown in table 1. The additional nucleotide sequence was added in the primers in order to generate DNA amplicons flanked by desirable restriction enzyme (AhdI) site, which would ultimately assist in cloning these DNA fragments into cloning vector system with the same restriction sites.

HeLa cell culture was successful in yielding sufficient population of cell to be used for isolating RNA. The percentage viability test showed that the culture conditions were good and the cells were 100% viable up to 6 hours post culture. Thereafter, the cells started dying which may be attributed to the lack of sufficient nutrients, lack of growth space and the production of toxic by-products by the cells. RNA isolation and optimization of the process was major step. We have tried to isolate RNA from different batches of the cells, with different number of cells and from different cell lines that were available. The results shows that the amount of RNA isolated from HeLa cells were higher as compared to A549 cells. This was performed in duplicate and average was taken. The difference in amount of RNA isolated may be due the difference in generation time of the two different cell lines which is short for HeLa. Similarly the RNA recovery was found to be increasing corresponding to increasing cell counts, which was obvious.

6. CONCLUSION

Palmitoylation of protein molecules is a major raft-targeting signal. Making use of this study we explored the relation between palmitoylation and membrane raft association of SNARE protein SNAP-23. Though the bioinformatic approach has helped in finding this relationship to some extent, it remains to be tested experimentally in the laboratory. Fusion protein SNAP-23 that contains a segment of non-raft associated protein that guide the fusion protein to non-raft region is believed to help in studying the importance of targeting of SNAP-23 to membrane raft and mediating membrane fusion. The relationship between palmitoylation and lipid raft association of SNAP-23 helps in finding the mechanism of membrane fusion and exocytosis at the raft region of the plasmamembrane. The mechanism of signaling and membrane association of SNAP-23 was not known clearly till date and this study has tried to find the answer to some extent.

The fusion protein that we have tried to create in-silico should be located to the non-raft region of the membrane. This is believed to occur because we have selected the proteins from non-raft regions and absence of any post-translational modifications in them would not signal these mutated proteins to their original site i.e., Lipid rafts. The goal of this bioinformatic approach was to create a fusion protein of SNAP-23 which will help in exploring the role of post-translational modifications of these proteins in anchoring and association with lipid-rafts where regulated exocytosis mediated by SNARE proteins is found to take place. This study, though a pioneering work in creating a fusion protein, will pave a definite pathway in creating the desirable chimera protein which will help in solving the question about the relationship between lipid-rafts and regulated exocytosis events mediated by SNARE proteins specifically SNAP-23. As per the analysis it was concluded that the fusion protein that were constructed would target the non-rafts region of the plasma membrane rather than membrane rafts regions and ultimately inhibits SNARE complex regulated exocytosis events.

It is well known that exocytosis is one of the major events that help various immune cells as well as secretory cells to render their function. Mast cell degranulation in allergic reaction is one of the most widely known events that take place by uncontrolled exocytosis of mast cell. So, if the process of exocytosis by mast cells can be controlled by modifying SNAP-23 then there is a possibility of decreasing allergic conditions caused by histamine, leukotriene, etc (released by mast cell degranulation) by pharmacological approach in future.

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8. APPENDIX

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

NaCl : 8 gm
$Na_2HPO_4.2H_2O$: 1.44 gm
KCl : 0.2 gm
KH_2PO_4 : 0.2 gm
pH was maintained to 7.3 to 7.4 with HCl

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

Glucose : 1.0 gm
HEPES : 2.50 gm
$NaHCO_3$: 2.60 gm
L-Glutamine : 0.30 gm
RPMI (SIGMA ALDRICH, USA) : 10.40 gm
Gentamycin : 120 mg
(pH- 7.0)

❖ **SOC medium (Super Optimal Broth with Catabolite Repression)**

Tryptone : 2 gm (2%)
Yeast extract : 500 mg (0.5%)
NaCl : 50 mg (10 mM)
KCl : 18.6 mg (2.5 mM)
$MgCl_2$: 95.2 mg (10 mM)
Glucose : 360 mg (20 mM)
The final volume was made up to 100 ml with MQ H_2O, filter sterilized and stored at 4^oC.

❖ **Solution-I**

50 mM glucose
25 mM TrisCl (pH-8.0)
10 mM EDTA (pH-8.0)
This was stored after autoclaving for 15 min (10 lb/in^2), 1 liquid cycle & stored at 4°C.

❖ **Solution-II (FreshlyPrepared)**

0.2 N sodium hydroxide
1% SDS

❖ **Solution-III**

5M Potassium acetate : 60 ml
Glacial acetic acid : 11.5 ml
Distilled water : 28.5 ml

❖ **STE solution**

0.1 M Sodium chloride
10 mM TrisCl (pH-8.0)
1 mM EDTA (pH-8.0)

❖ **TE buffer**

10 mM Tris HCl (pH-8.0)
10 mM EDTA (pH-8.0)

❖ **5X TBE buffer** :

54 g Tris Base
27.5 g Boric acid
20 ml 0.5 M EDTA (pH-8.0)
Make final volume upto 1 liter (final pH-8.0)

❖ **6X Gel loading dye**

10 mM Tris (pH-8.0)
0.03% Bromophenol blue
60 % Glycerol
60 mM EDTA

❖ **10X Formaldehyde Agarose Gel buffer:**

200 mM 3-[N-morpholino] Propanesulfonic acid (MOPS)
50 mM Sodium acetate
10 mM EDTA
(pH to 7.0 with NaOH)

❖ **1X Formaldehyde Agarose gel running Buffer:**

100 ml 10X formaldehyde agarose gel buffer
20 ml 37% (12.3M) formaldehyde
880 ml RNAse free water

- **<u>5X RNA loading dye:</u>**

  16 µl saturated aqueous bromophenol blue solution
  80 µl 500mM EDTA, pH 8.0
  720 µl 37% (12.3M) formaldehyde
  2 ml 100% glycerol
  3084 µl formamide
  4mL 10X formaldehyde agarose gel buffer
  RNAse free water to 10ml

Photograph 1: Investigator Operating PCR machine

Photograph 2: Investigator (2nd from left) with Supervisor **(2nd from right) and Senior Scholars** at SLS, JNU, New Delhi