Univerzita Karlova v Praze

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Katedra biochemie

Bc. Barbora Volfová

Funkční analýza fosforylace syntaxinu 16 za použití kvasinkového modelu

Functional analysis of syntaxin 16 phosphorylation using yeast as a model

Diplomová práce

Vedoucí práce: **Prof. RNDr. Gustav Entlicher, CSc.**

Konzultanti: **RNDr. Jiří Novotný, DSc.**

Scott G. Shanks, PhD.

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Charles University in Prague

Faculty of Science

Department of Biochemistry

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Diploma Thesis

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Consultants: **RNDr. Jiří Novotný, DSc.**

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Declaration

I declare that the work presented in this thesis has been carried out by me, unless otherwise stated. It is entirely of my own composition and has not, in whole, or in part been submitted for any other degree.

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Abstrakt

Mechanismus fúze intracelulárních membrán v eukaryotických buňkách zahrnuje několik skupin proteinů, včetně rodiny receptorů pro záchytný protein rozpusného N-ethylmaleinimid-senzitivního-faktoru (SNARE) a Sec1/Munc-18 příbuzných proteinů (SM proteinů). Je známo, že transport v buňkách je evolučně konzervovaný od kvasinek po člověka. Proto pro usnadnění výzkumu, můžeme použít jednoduché eukaryotní buňky Saccharomyces cerevisiae. Savčí SNARE protein syntaxin 16 má známý funkční kvasinkový homolog, protein Tlg2p, který byl použit v této studii jako model pro studium vlivu fosforylace na funkci syntaxinu 16. Také jejich vazebné partnery, SM proteiny mVps45p (savčí) a kvasinkový Vps45p, jsou homologní.

Fosforylace SNARE proteinů je známá jako možný způsob regulace fúze membrán. Odstranění jednoho z možných fosforylačních míst na Tlg2p - serinu 90 významně ovlivňuje exocytické a endocytické pochody. Práce prezentovaná v této studii ukazuje některé fenotypy mutant založených na tomto fosforylačním místě proteinu Tlg2p. Těmi mutantami jsou S90A (nefosforylovatelná – mutace serinu na alanin) a S90D (fosfomimetická – mutace serinu na asparagovou kyselinu – kyselá karboxylová skupina napodobuje fosfátovou skupinu). Bylo zjištěno, že fosforylace proteinu Tlg2p na serinu 90 neboli mutace Tlg2p-S90D může hrát jistou roli v ochraně proteinu Tlg2p před degradaci nezávislou na Pep4p pravděpodobně pomocí proteazomu.

V nepřítomnosti genu PEP4, kdy je zabráněno vakuolární degradaci, je účinek proteazomu vidět jasněji. Protein Pep4p je vakuolární proteáza v kvasinkách zodpovědná za degradaci mnoha bílkovin. Ostatní výsledky také ukazují, že fosforylace Ser90 na Tlg2p neboli S90D fosfomimetická mutace může stabilizovat interakci mezi proteiny Tlg2p a Vps45p, a tím stabilizovat množství Tlg2p v buňce. Bylo také prokázáno, že fosforylace nemá žádný dopad na transport karboxypeptidázy Y a na citlivost na solný/osmotický stres.

Dále jsme také ukázali, že je možné připravit in vitro hybridní SNARE komplex obsahující savčí syntaxin 16 a kvasinkové proteiny Tlg1p, Vti1p a Snc2p, a že tento komplex je schopen vázat protein Vps45p v podobném množství jako kvasinkový SNARE complex s proteinem Tlg2p jakožto syntaxinovou částí komplexu. Tato zjištění mohou být velmi užitečná pro budoucí výzkum syntaxinu 16 a samotného transportu v savčích buňkách. (In English)

Klíčová slova: Fúze membrán, SNARE, Syntaxiny, Tlg2p, Sec1p/Munc18, Vps45p, Endocytoza

Abstract

Mechanism of fusion of intracellular membranes in eukaryotic cells involves several protein families including soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins and Sec1/Munc-18 related proteins (SM proteins). It is known that the transport is evolutionary conserved from yeast to man. Therefore for facilitating of the research, we can use simple eukaryotes *Saccharomyces cerevisiae*. Mammalian SNARE protein syntaxin 16 has a yeast homologue Tlg2p which is used in this study as a model for studying affects of phosphorylation to the syntaxin 16 function. Also their binding partners, SM proteins mVps45p (mammalian) and yeast Vps45p are homologous.

Phosphorylation of SNARE proteins is known as a possible way of regulation of membrane fusion. Abolishment of one of the putative phosphorylation sites in Tlg2p protein, serine 90 leads to dominant effects on the exocytic and endocytic pathways. The work presented in this study shows some phenotypes of mutants based on this phosphorylation site of protein Tlg2p. Those mutants are S90A (cannot be phosphorylated) and S90D (phosphomimetic – acid carboxyl group mimics phosphate group). It was revealed that the phosphorylation of Tlg2p protein at serine 90 or the mutation Tlg2p-S90D may play some role in protecting Tlg2p protein from non-Pep4-dependent degradation, possibly by the proteasome. In the absence of *PEP4*, when the vacuolar degradation is prevented, the effect of the proteasome can be seen more clearly. Protein Pep4p is a vacuolar protease in yeast responsible for degradation of many yeast proteins. Other results also suggest that phosphorylation of Ser90 on Tlg2p or the S90D phosphomimetic mutation may stabilise the interaction between Tlg2p protein and Vps45p protein, and therefore stabilise the levels of Tlg2p in cell. It was also shown that phosphorylation has no impact on trafficking of carboxypeptidase Y phenotype and salt/osmotic stress sensitivity phenotype.

We also showed that it is possible to prepare in vitro hybrid SNARE complex containing mammalian protein syntaxin 16 and yeast proteins Tlg1p, Vti1p and Snc2p and that this complex is able to bind similar amounts of Vps45p protein as yeast SNARE complex with Tlg2p protein as a syntaxin part. These findings may be very usefull for future research of syntaxin 16 and the trafficking in mammalian cells. (In English)

Key words: Membrane fusion, SNARE, Syntaxins, Tlg2p, Sec1p/Munc18, Vps45p, Endocytosis

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Abbreviations

APS	ammonium persulfate
bp	basepairs
CAPP	ceramide-activated protein phosphatase
cat #	catalogue number
cDNA	complementary deoxyribonucleic acid
C. elegans	*Caenorhabditis elegans*
cyto	cytosolic
dNTP	deoxyribonucleotide triphosphate
Δ	deletion
DTT	dithiothreitol
E. coli	*Escheria coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EDTA-Na	ethylenediaminetetraacetic acid disodium salt
ER	endoplasmic reticulum
EST	expressed sequence tag
g	gravitational force
GLUT4	glucose transporter 4
GST	glutathione S-tranferase
GSV	GLUT4 storage vesicle
HA	hemagglutinin
HAc	acetic acid
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazine]ethanesulfonic acid
His	histidin
HRP	horseradish peroxidase
IgG	immunoglobin G
IPTG	isopropyl-β-galactosidopyranoside
KOAc	potassium acetate

LSB	Laemmli sample buffer
M	molar
met	methionine
mRNA	messenger RNA
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
NSF	N-ethylmaleimide-sensitive factor
OD600	optical density at 600 nm
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween20
PCR	polymerase chain reaction
PKA	protein kinase A
PrA	protein A
RNA	ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisce*
SA	mutant S90A
SD	mutant S90D
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	Sec1p/Munc18
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
TAE	Tris-acetic acid-EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween20
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N’,N’, - tetramethyl ethylene diamine
TGN	*trans*-Golgi network
Tlg2p	t-SNARE of the late Golgi compartment 2

TM	transmembrane
TMD	transmembrane domain
Tris	2-Amino-2-hydroxmethyl-propane-1,3-diol
t-SNARE	target SNARE
TST	tris saline with Tween20
ura	uracil
VAMP	vesicle associated membrane protein
v-SNARE	vesicle SNARE
VPS	vacuolar protein sorting
WT	wild type
YPD	yeast peptone dextrose

1. Introduction – Transport and trafficking in eukaryotic cells

A defining characteristic of all eukaryotic cells compared to prokaryotic cells is the presence of membrane-bound compartments inside the cell. These compartments known as organelles interact with each other and with the cell environment. Transport between those compartments is essential for the life of the cell. The transport proceeds using small membrane-enclosed sacs called vesicles via two major pathways: the outwards, exocytic-secretory pathway carries material synthesized in the cell to the plasma membrane and the extracellular medium, while the inwards, endocytic pathway carries material from the cell surface into the cell interior (Fig. 1).

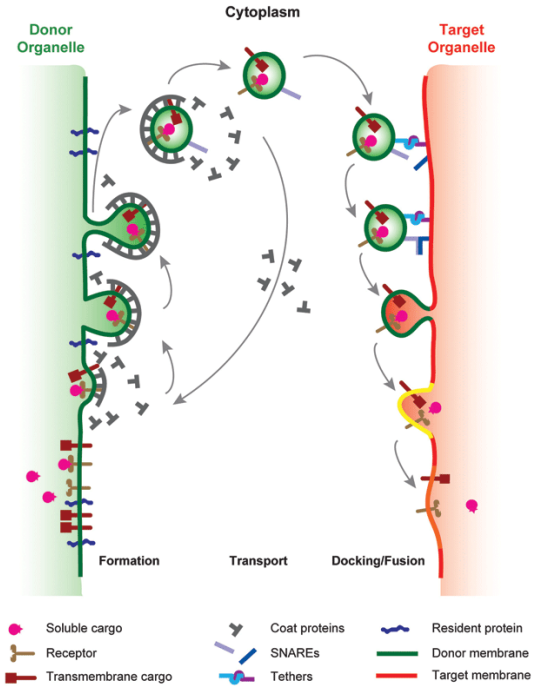

Figure 1: Overview of endocytic and exocytic-secretory pathways in eukaryotic cells (modified from [1])

This is a highly specific mechanism that ensures the correct delivery of molecules from one intracellular membrane bound compartment to another [2], [3]. Mechanism of fusion of intracellular membranes in eukaryotic cells involves several protein families including SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor)

proteins, Sec1/Munc-18 related proteins (SM proteins), Rab proteins [4], [5], tethering factors [6], [7], [8], [9] and others.

1.1. SNARE proteins

SNARE (soluble NSF attachment protein receptor**)** proteins were firstly discovered independently in yeast cells and neurons (reviewed in [10], [11]). On the basis of their localization, SNAREs were classified into two groups: t-SNARE (localized to the target membrane) and v-SNARE (localized to the membrane of the trafficking vesicle) [12]. However this classification system is not exact, because homeotypic fusion of two yeast vacuoles containing the same sets of SNARE proteins is symmetric. Thus a second nomenclature system has been developed which categorizes SNAREs in terms of a single key residue that is usually either arginine (R-SNAREs = v-SNAREs) or glutamine (Q-SNAREs = t-SNAREs) [13].

SNARE proteins are very important proteins in membrane fusion. For the fusion of two membranes are required in general four SNAREs. The intracellular membrane fusion reactions involve one R-SNARE and three Q-SNAREs [14], [15]. In the case the fusion system is asymmetric, R-SNAREs are usually contributed by the vesicle and Q-SNAREs by the target organelle. Assembled SNARE complexes that bridge two membranes are called *trans*-SNARE complexes. Membrane fusion converts these *trans* complexes to *cis* complexes, complexes in which all the SNAREs are associated with the same membrane [16].

The SNARE complex was first characterized in neurons when an integral membrane protein, syntaxin 1, in association with synaptosomal protein of 25 kDa (SNAP-25) and vesicle-associated membrane protein (VAMP) were co-immunoprecipitated as a protein complex with α-SNAP (soluble NSF attachment protein) and NSF (N-ethylmaleimide-sensitive factor) (see 1.3.) [12], [17], [18].

The structure of SNARE proteins is characterized by the presence of SNARE motif that has approximately 60 – 70 residues in length, is predicted to form a coiled-coil structure and is located immediately adjacent to a C-terminal transmembrane anchor [19]. There are two exceptions from the rule written above: first, in a few cases, two SNARE motifs can be contained within the same polypeptide chain and second, in other cases the SNARE does not contain a C-terminal transmembrane anchor. The SNARE motif has repeating heptad pattern of hydrophobic residues with all side chains localized on the same face of the helix. SNARE

motifs during the fusion assemble into parallel four-helix bundles stabilized by the burial of these hydrophobic helix faces in the bundle core [20], [21], [22].

1.1.1. SNARE hypothesis

In 1993, Rothmann and colleagues postulated 'The SNARE hypothesis', a model in which each transport vesicle contains one or more members of the v-SNARE superfamily, and every target compartment in a cell contains one or more members of the t-SNARE superfamily. Specificity in membrane transactions would be assured by the unique and non-overlapping distribution of v-SNAREs and t-SNAREs among the different vesicles and target compartments [12].

Lately it was shown that SNARE pairing is less specific that was previously assumed [23]. It was shown that SNARE interactions are promiscuous. In addition to typical SNARE-pair interactions, between SNAREs of different families was also observed a number of atypical SNARE-pair interactions as well as homo-multimerization of SNAREs [24]. Thus, membrane fusion specificity is perhaps not due to a single mechanism but rather involves several different layers of regulation [25].

1.1.2. Syntaxins

An important family of SNARE proteins is the syntaxin family. They are classified as t-SNARE proteins that can be found usually on the target membrane. Syntaxins 1A and 1B were first syntaxins that were described. They were described as two 35 kDa proteins that interact with the synaptic-vesicle protein called synaptogamin [26]. The syntaxin family consists of 15 proteins expressed in mammals (syntaxin 1A, syntaxin 1B, syntaxin 2, syntaxin 3, syntaxin 4, syntaxin 5, syntaxin 6, syntaxin 7, syntaxin 8, syntaxin 10, syntaxin 11, syntaxin 12/13, syntaxin 16, syntaxin 17, syntaxin 18) and 7 in yeast (Sso1p, Sso2p, Ufe1p, Sed5p, Tlg2p, Pep12p, Vam3p). Almost all mammalian syntaxins (except of syntaxin 11) are type II transmembrane proteins anchored by carboxy terminal tail. There are several other hydrophobic domains with the potential to form coiled-coil structures. The most important domain which is conserved in all syntaxins is the approximately 60-residue-long membrane-proximal coiled-coil SNARE domain (also called H3 region).

The SNARE domain of syntaxins mediates its interactions with other SNARE domains of t-SNAREs. It forms a t-SNARE complex at the target membrane which can then interact with SNARE domain of SNARE protein on the vesicle membrane (v-SNARE), to form the core fusion complex.

The amino terminus of syntaxins contains another characteristic domain called Habc domain. It was firstly observed in syntaxin 1A but it is conserved in all plasma membrane syntaxins (all excluded syntaxin 11). It is an autonomously folded domain and the structure consists of an up-and-down three-helix bundle with a left-handed twist [27].

Separately, syntaxins are in so called "closed" conformation. In this conformation, the N-terminal part of the Habc domain interacts with the C-terminal domain of cytoplasmic H3 region, which contains SNARE domain. It means that the SNARE domain of syntaxins is hidden by the Habc domain thus syntaxin is not able to interact with other SNARE partners and form the SNARE core complex [28]. It is known that in this conformational state, syntaxins are stabilized by so called Sec1/Munc18 (SM) proteins. To form a core complex, syntaxins have to convert into an "open" conformation.

Schematic diagram of syntaxins can be seen on Fig. 2

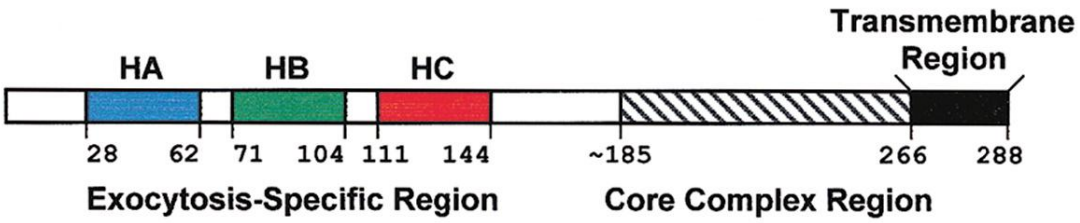

Figure 2: Schematic diagram of syntaxins representing the key domains. The residue numbers are specific for syntaxin 1A (modified from [27])

1.2. SM proteins

Next protein family which is important for transport and trafficking in eukaryotic cells are the Sec1/Munc18 (SM) proteins. It is a highly conserved family and the homology between individual members of SM proteins is throughout the entire sequences.

SM proteins are cytosolic proteins of ≈600-700 residues that folds into an arch-shaped "clasp" structure [29]. In contrast to the many SNARE protein isoforms in vertebrates and yeast, only seven SM proteins are expressed in vertebrates (Munc18-1, Munc18-2, Munc18c, Sly1, Vps45, Vps33a and Vps33b), and only four SM proteins in the yeast (Sec1p, Sly1p, Vps45p and Vps33p) [30].

The first SM protein that was identified was Unc18 and it was discovered genetically in *C. elegans* [31]. The first mammalian SM protein that was discovered was Munc18, homolog of Unc18, by its ability to bind with high affinity to syntaxins [32].

Members of this family have been implicated in vesicle targeting in a variety of intercompartmental transport events [33].

The Sec1/Munc18 (SM) family plays an essential role in regulating membrane transport [34]. Disruption or deletion of any of the four SM proteins in *S. cerevisiae* causes a block in vesicle transport [34]. SM proteins function is to regulate membrane fusion by interacting with their cognate syntaxins.

1.2.1. Regulation of membrane fusion

Based on data from binding studies, it was suggested that SM proteins may be involved in the functional regulation of syntaxins. All of the known SM proteins appear to interact specifically with syntaxin family members, raising the possibility that all syntaxin family members may require an SM protein for function [34].

It is not known how exactly SM proteins cooperate with SNARE complexes in fusion but it seems that SM proteins may likely act as a chaperone like catalysts for SNAREs, which in turn are catalysts for membrane fusion [35].

1.2.1.1. Binding of SM proteins to syntaxins

SM proteins interact with SNAREs in different ways [35]. It was discovered that SM proteins can bind to their cognate syntaxins by three different modes.

The Mode 1 interaction: SM protein binds tightly to a closed conformation of syntaxin preventing transition of syntaxin to its “open” conformation, Mode 2 interaction: binding of SM protein to the extreme amino terminus of their appropriate syntaxins, Mode 3 interaction: binding of SM protein to the assembled complexes of the t-SNAREs and more strongly to the full SNARE complex, but not to its monomeric syntaxin [36].

The Mode 1 was firstly observed on binding partners syntaxin 1a and SM protein Sec1. Neuronal nSec1 is an arch-shaped molecule consisting of three domains and syntaxin 1a forms four-helix-bundle structure that fills the central cavity of nSec1. In the nSec1-syntaxin 1a complex, residues of H3 that are crucial to form SNARE complex contact either nSec1 or

Habc, implying that nSec1-bound syntaxin 1a must undergo a large conformational change in order to form the SNARE complex [29].

The Mode 2 was firstly discovered when binding of yeast syntaxin Sed5p to its cognate SM protein Sly1p was studied. The crystal structure of Sly1p in complex with a 45 residue peptide derived from the N-terminus of Sed5p shows that Sed5p interacts predominantly with domain I of the arch-shaped Sly1p [37]. It was found that the most important residue in the Sly1p-Sed5p interaction is Phe10, which binds into a highly conserved hydrophobic pocket on Sly1p [37]. The crystal structure of Sly1p in complex with the N-terminal 45 residues of Sed5p revealed that Phe10 of Sed5p interacts with Sly1p via a hydrophobic pocket on the surface of domain I of the SM protein, Sly1p.

An example of the Mode 3 is binding between Sso1p and Sec1p. It was observed that Sec1p co-precipitates all three components of the exocytic SNARE complex, Ssop, Sec9p, and Sncp [38]. Several observations suggest that Sec1p preferentially co-precipitates with SNAREs assembled into SNARE complexes. The ratio of Ssop to Sncp in Sec1p immunoprecipitations resembles the 1:1 ratio of these two proteins in purified SNARE complexes [38]. The results of mixing experiments and binding studies with purified SNARE complexes established that the association of Sec1p with preassembled SNARE complexes does not require an interaction between Sec1p and SNARE components before the complex assembly [38]. It is not known whether Sec1p preferentially binds to complexes of SNARE proteins on opposing membranes (*trans*-SNARE complexes) or on the same membrane (*cis*-SNARE complexes)[39].

1.3. Membrane fusion overview

Membrane fusion can be divided into 5 steps. First the vesicle has to approach the target membrane. The first phase is called the tethering and docking step. In this stage vesicles are approaching the target membrane are recruited to the appropriate place on the target membrane by tethering and docking factors. The next step is the formation of a trans-SNARE core complex. The core complex is made by 4 α-helices from 4 SNARE domains, 3 are from t-SNAREs and 1 from the v-SNARE. The t-SNAREs are also called Qa, Qb and Qc. The Qa protein is syntaxin. After the trans-SNARE complex formation follows the membrane fusion. The energy provided during the formation of the SNARE complex by the zippering of the helices is $35k_BT$. It is not sufficient for complete membrane fusion but it is enough for

hemifusion [42]. For complete lipid bilayer fusion approximately 50 – 100 k_BT is required, and it is thought to come from the formation of 3 or more trans-SNARE complexes between the membranes [42], [43], [44]. After the distance between two bilayers is sufficiently reduced, hemifusion occurs, followed by distal leaflet membrane breakdown, resulting in the opening of fusion pore. Finally, the fusion pore expands, causing full content mixing and membrane relaxation [45]. After the fusion, the trans-SNARE core complex is transformed into a cis-SNARE complex with all SNARE proteins on one membrane. The disassembly of SNARE complex is mediated by the ATPase NSF and an adaptor protein, α-SNAP. The final step is recycling of SNARE proteins (Fig. 3).

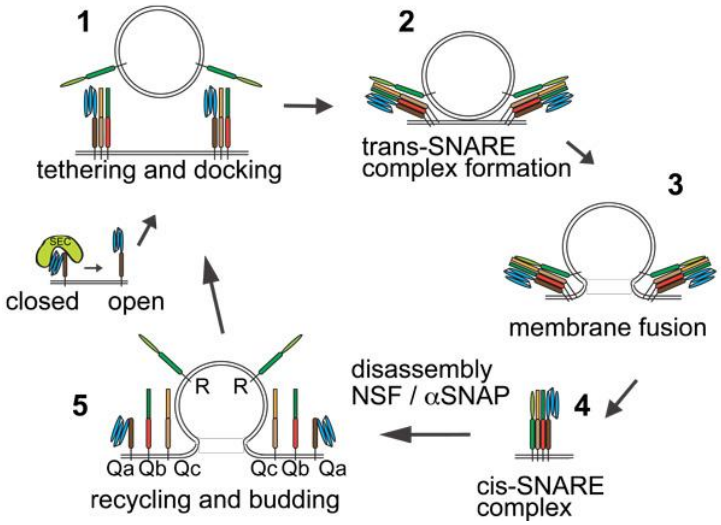

Figure 3: Membrane fusion overview: 1. tethering and docking of vesicle, 2. trans-SNARE core complex formation from 3 t-SNAREs and 1 v-SNARE. 3. membrane fusion, 4. cis-SNARE complex formation, 5. disassembly of SNARE complex and recycling of SNAREs; R: v-SNARE (modified from [46])

1.4. Membrane fusion from yeast to mammals

It is known that membrane budding and fusion occur in all eukaryotic cells. The mechanism of the processes was initially studied in mammalian neurons and in yeast *S. cerevisiae.*

At the beginning it appeared that vesicle transport in these two cell-types is quite different. Secretion in yeast is a constitutive process, for example, whereas synaptic vesicle exocytosis is tightly regulated by intracellular Ca^{2+} concentration [11]. Another difference occurs in time of exocytosis (in the nerve terminal it proceeds faster than in other secretory systems). Further studies have shown that there are many similarities between the two

systems. It was demonstrated that the yeast *SEC18* gene product can replace mammalian NSF in a cell free assay that reconstitutes membrane transport [47].

In neurons, the fusion of a synaptic vesicle with the plasma membrane requires the following proteins: synaptobrevin (also known as VAMP), syntaxin and the synaptosomal associated protein of relative molecular mass 25 000 Da (M_r 25K; SNAP-25) [11]. These proteins are structurally related to known yeast proteins many of which are required for the targeting and/or fusion of transport vesicles with their acceptor compartment at different stages of the secretory pathway [11].

Homologues of mammalian proteins involved in fusion have continued to be identified in yeast. This important piece of knowledge has established yeast as a good model for studying transport and trafficking in eukaryotic cells.

1.4.1. Membrane fusion in mammals

Many SNAREs were already identified and localized to specific subcellular locations of eukaryotic cell. According to what is known, there is a relatively large number of SNAREs to distinguish between the various vesicle trafficking steps. All SNAREs have distinct subcellular localizations [48].

The human genome sequence revealed higher numbers of SNAREs, with 35 so far identified. These seem largely to be tissue-specific isoforms, with brain-specific forms particularly well represented [14], [49].

1.4.1.1. Syntaxin 16

Syntaxin 16 was first cloned and sequenced by Simonsen and colleagues [50]. Syntaxin 16 was identified by searching the expressed sequence tag (EST) database for sequences which showed similarities to the C-termini of syntaxins [50].

PCR primers were designed to one EST which contained a 246 bp region that showed homology to the syntaxin family. This allowed amplification of full-length cDNA from brain cDNA libraries and HeLa cell cDNA libraries. The two longest PCR products generated from the HeLa plasmid cDNA library were named syntaxin 16A and syntaxin 16C. They were almost identical except syntaxin 16A contained a 161 bp insertion and a 12 bp deletion [50]. Syntaxin 16C also contains an in-frame stop codon in place of the syntaxin 16A insertion,

which results in the translation of a C-terminally truncated protein lacking the SNARE and TM domain of syntaxin 16A [50].

The longest PCR product obtained from the brain cDNA plasmid library, named syntaxin 16B, was identical to syntaxin 16A but it contained an additional 63 bp insertion [50].

The tissue distribution of syntaxin 16, was determined on northern blots containing mRNAs from the human tissues, heart, brain, placenta, skeletal muscle, kidney and pancreas using a probe that recognized all three splice variants of syntaxin 16, however only one band, of approximately 5 kb was detected in all of the tissues tested [50]. The explanation for this was that the syntaxin 16 mRNAs must contain large untranslated regions and suggest that the sizes of the mRNAs are too similar to be resolved.

Confocal immunofluorescence microscopy, showed that syntaxin 16A co-localises with the Golgi marker β COP and also partially co-localises with the TGN marker, mannose-6- phosphate receptor [50]. Syntaxin 16A also displayed a small amount of co-localisation with the ER marker, calreticulin. However treatment of cells with cycloheximide demonstrated that syntaxin 16A is only initially associated with the ER membrane prior to reaching its final destination at the Golgi [50].

At the same time, another group also found a human EST potentially coding a novel syntaxin-like molecule [51]. They started to use the name human syntaxin 16 (syntaxin 16H). Syntaxin 16H is highly homogenous, but not identical to, both syntaxin 16A and syntaxin 16B. Syntaxin 16C is identical to the N-terminal 115 amino acids of syntaxin 16H, but the predicted coding sequence of syntaxin 16C ends at amino acid 116 [51].

Tang et al. [51] also assessed syntaxin 16H tissue distribution. They did multiple tissue Northern blots with the full length cDNA, which revealed a single transcript of about 6.5 kb which has a fairly ubiquitous expression, being slightly more enriched in heart and pancreas. The ubiquitous expression of syntaxin 16H suggests that it has a general function which is not restricted to particular tissue or cell type [51].

Localization of syntaxin 16H to the Golgi apparatus suggests that it participates in a vesicular transport process in this organelle [51].

Next investigation of syntaxin 16 by the group of Dulubova (2002) [52] provided further data on the function of this protein. They found that although syntaxin 16 mRNA is expressed at fairly uniform levels in most tissues (as written above), the levels of syntaxin 16 protein expressed are selectively high in brain. These results suggest that the synthesis and/or

stability of syntaxin 16 are regulated post-transcriptionally, and that syntaxin 16 has a specific, as yet uncharacterized, brain function [52].

It was also demonstrated that syntaxin 16C represents the truncated version of syntaxin 16 where the only intact functional region is the N-terminal peptide sequence [52].

By co-immunoprecipitation it was shown that syntaxin 16 forms a functional t-SNARE core complex with syntaxin 6 and Vti1a, and that syntaxin 6/syntaxin 16/Vti1a interacts with the v-SNARES VAMP3/cellubrevin or VAMP4 [53].

1.4.1.1.1. Syntaxin 16 is involved in trafficking of GLUT4

The uptake of glucose into mammalian cells involves a family of transport proteins called GLUTs which act as shuttles to move sugar across the cell surface. These polytopic membrane proteins form an aqueous pore across the membrane through which glucose can move [54].

GLUT4, which is expressed primarily in muscle and fat cells, is found in a complex intracellular tubulovesicular network that is connected to the endosomal–*trans*-Golgi network (TGN) system [54]. The translocation of GLUT4 to the plasma membrane is stimulated by increased amount of insulin in blood. In the absence of stimulation, GLUT4 is almost completely excluded from the plasma membrane but the addition of insulin, or exercise in the case of muscle cells, causes GLUT4 to shift from its intracellular location to the plasma membrane [54].

Several observations indicate that GLUT4 has a crucial role in whole-body glucose homeostasis. First, insulin-stimulated glucose transport is an important rate-limiting step for glucose metabolism in both muscle and fat tissue, and it is severely disrupted in type II diabetes [55]; second, disruption of GLUT4 expression in mice results in insulin resistance [56]; and over-expression of GLUT4 ameliorates diabetes in the DB/DB MOUSE model [57].

Although the majority of GLUT4 is found in small tubulovesicular elements in muscle and adipocytes it was found that a portion of GLUT4, which does not represent newly synthesized protein GLUT4, is present in the TGN of both muscle and fat cells, and that GLUT4 recycles rapidly between the TGN and endosomes [54].

In 2002, Bryant and James proposed a model of GLUT4 transport based on previous observations. The main feature of this model is that GLUT4 is selectively targeted to an intracellular transport loop between the TGN and endosomes. The entry of GLUT4 into this

intracellular, seemingly futile, cycle probably excludes it from the cell surface recycling pathway [54].

After insulin stimulation, the majority of GLUT4 is localized to a specialized compartment that is analogous to small secretory vesicles present in neuroendocrine tissues, termed GLUT4 storage vesicles (GSVs) [54].

It was shown that GLUT4 recycles via a TGN sub-domain enriched in syntaxins 6 and 16 [58] back into the insulin-sensitive compartment, suggesting that these t-SNAREs may be part of a complex which regulates GLUT4 sorting.

Syntaxin 6 exhibits a significant degree of colocalization within GLUT4 vesicles with >85% of the cellular complement of syntaxin 6 is found within GLUT4 vesicles. Syntaxin 6 also exhibited insulin-stimulated translocation to the plasma membrane which suggests that at least a portion of intracellular syntaxin 6 is present within the fraction of GLUT4-containing vesicles that translocates to the cell surface upon insulin stimulation [59]. Further studies showed that traffic of GLUT4 between the endosomes and GSVs is regulated by/requires syntaxin 6 and if the function of syntaxin 6 is impaired, GLUT4 sorting to the GSVs is perturbed [59]. It was also shown that syntaxin 16 may act as the cognate t-SNARE for this transport step because both syntaxin 6 and syntaxin 16 exhibit insulin-dependent translocation to the cell surface, presumably because of their presence in GLUT4 vesicles in either (or both) of endosomes and GSVs [59].

In the study of Proctor et al. (2006), it was demonstrated that syntaxin 16 was involved in the intracellular trafficking of GLUT4. It was proposed that syntaxin 16 functions to control the entry of GLUT4 into the slowly recycling pathway (or to control the exit of GLUT4 from the fast recycling endosomal system) into the GSV compartment, and thus regulate the insulin responsiveness of adipocytes [60].

1.4.2. Membrane fusion in yeast

As described earlier, membrane fusion is evolutionarily conserved from yeast to mammals. This makes yeast an extremely useful model for characterizing components of membrane fusion through genetics, biochemistry and bioinformatics.

One of the most important components mediating fusion between vesicle and target are called SNARE proteins. SNAREs mediate transport of vesicles across the whole cell. Transport between different compartments is mediated by different SNARE proteins. In Fig. 4 (page 23), you can see subcellular location of yeast SNAREs.

Recent genome-wide analyzes suggest that 24 SNAREs are present in yeast. Most SNAREs (20 out of 24 in yeast) are tail-anchored proteins: type II membrane proteins with a C-terminal segment of polypeptide that serves as a membrane anchor [61].

The biggest family of SNARE proteins is the syntaxin family. In yeast, there are 6 different syntaxins found. The latest one discovered is Tlg2p (t-SNARE of the late Golgi 2).

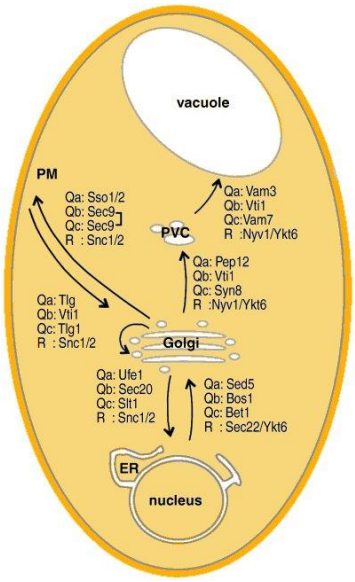

Figure 4: Subcellular location of yeast SNAREs. Each of the SNAREs contributes a helical segment to at least one SNARE pin, for anterograde or retrograde traffic between endoplasmic reticulum (ER) and Golgi, within the Golgi stacks or from the Golgi to the prevacuolar compartments (PVC) and vacuole or to the plasma membrane (PM). Endocytic transport makes use of the Golgi SNAREs to deliver cargo to an endosomal compartment equivalent to the PVC; R – v-SNARE (modified from [62]).

In the present study we have been investigating the t-SNARE protein Tlg2p which mediates trafficking in the Trans Golgi network (TGN).

1.4.2.1. Tlg2p

Tlg2p is a member of the yeast syntaxin family. It is type II transmembrane protein composed of 396 amino acids long with a domain structure typical of syntaxins [52]. It has a Habc domain (residues 70-187), a SNARE domain (residues 253-307) and a transmembrane domain (residues 317-334) [52]. Tlg2p is unique among known syntaxins in possessing a sizeable hydrophilic domain of 63 amino acids that is C-terminal to the membrane spanning

region and apparently nonessential for Tlg2p function. The presence of this domain does not alter the overall topology of the rest of the protein [63].

Tlg2p is involved in controlling membrane fusion and protein trafficking through the endocytic system [63]. It cycles between the late Golgi and the endosome. The evidence of the intracellular localization of Tlg2p is that it co-localizes with the TGN marker Kex2p and also co-fractionates with a second TGN marker DPAP A [64]. The involvement of Tlg2p in endocytosis was confirmed using null-mutants lacking the *TLG2* gene (Δ *tlg2*) by following the fate of two established endocytic markers, α-factor and uracil permease, and that of a recently introduced endocytic tracer, positively charged Nanogold [65]. Tlg2p was found not to be involved in the secretory pathway.

The predicted TMD of Tlg2p is 17 amino acids long. Recent data indicate that long TMD (~25 amino acids) would play a critical role in plasma membrane localization, whereas for shorter TMDs, membrane localization depends on less easily definable physical properties, including amino acid composition or cytosolic signals. These would be important to define ER/Golgi or endosome/vacuolar localization [66]. The intracellular localization of Tlg2p is in agreement with the short length of its TMD [65].

It was shown that Tlg2p is nonessential protein for viability of yeast cells although depletion of Tlg2p causes some phenotype differences. The Δ*tlg2* disruptants are superficially normal and grow only slightly slower than the parental strain (WT) [64]. Depletion of Tlg2p causes missorting of a 15-20 % of carboxypeptidase Y, defects in endocytosis and failure to grow on high salt medium [63]. Although cells lacking Tlg2p have a sorting defect, their membrane fusion events required in transport from the late Golgi to the vacuole via the endosome/prevacuolar compartment are not blocked [63]. Another phenotype observed in Δ *tlg2* mutants was abnormal fragmentation of the vacuole [63], [64].

In addition, Tlg2p is required for normal endosome biogenesis [65], for efficient localization of casein kinases to the plasma membrane [67], for import of aminopeptidase I from the cytosol to the vacuole [68], for recycling of the SNARE Snc1p through early endosomes [69] and for TGN homotypic fusion [70].

It was discovered that phosphorylation of the Tlg2p modulates the ability to confer endocytic trafficking by inhibiting SNARE assembly [71]. They showed that the restoration of endocytosis by CAPP (ceramide-activated protein phosphatase) activation correlates with Tlg2p dephosphorylation and SNARE complex assembly. Also it was shown that endosomal Tlg2p appear to undergo the same type of regulation shown for the Sso exocytic t-SNAREs both *in vivo* and *in vitro* [72].

Therefore, putative protein kinase A (PKA) phosphorylation sites in *TLG2* gene were mutated in the same region as reported previously for Sso proteins. It was shown that mutation of a PKA site Ser79 (serine 79) which is located in the autoinhibitory domain (Habc domain) of Sso confers full exocytic functioning in the absence of CAPP activation [72].

It was shown that abolishment of putative PKA site of *TLG2* Ser90 (serine 90) leads to dominant effects on the exocytic and endocytic pathways [71].

SNARE phosphorylation may thus regulate membrane fusion events. However is not known how this important mechanism functions.

Tlg2p also interacts physically and functionally with Vps45p, the SM protein involved in TGN/early endosomal transport [68], [70], [73], [74].

1.4.2.1.1. SNARE complex containing Tlg2p

Co-immunoprecipitation experiments revealed that yeast syntaxin Tlg2p is associated with several others SNARE proteins involved in endocytosis and forms binary or ternary complexes containing various combinations of Tlg1p, Vti1p and Snc2p [63], [64], [75]. Vti1p is a light chain of the vacuolar t-SNARE, but it is also found in endosomes and Golgi [76], [77]. Snc2p, the v-SNARE normally used in fusion of the Golgi complex with the plasma [78], [79], [15] is also required for endocytosis [80]. Tlg1p was firstly identified as an endosomal protein [64] and is also required for endocytosis. Although it was originally classified as a syntaxin, its homology is actually closer to that of a light chain (non syntaxin) t-SNARE or a v-SNARE [19]. Thus all of these SNARE proteins are known to be involved in endocytosis and many participate in other trafficking steps as well [81]. It was also found that cells lacking Tlg1p or Vti1p display similar defects as cells lacking Tlg2p [64], [75].

It has been shown previously that functional yeast t-SNAREs marking the Golgi compartment, the plasma membrane and the vacuole are each composed of a distinct heavy chain from the syntaxin family and, depending on the particular membrane, one or two nonsyntaxin light chains [15], [82], [83]. The architecture of the endosomal t-SNARE further establishes the generality of this concept with Tlg2p as the heavy chain and Tlg1p and Vti1p as its two light chains, respectively, functioning exclusively with Snc2p as its v-SNARE [81]. It was checked whether this SNARE complex is functional in a liposome fusion assay and it was shown in two independent populations of liposomes that Tlg2p, Tlg1p Vti1p and Snc2p allowed fusion which means that these four SNARE proteins can form functional SNARE complex *in vitro* [81].

1.4.2.2. Vps45p

Genetic analysis of vacuolar protein sorting in *S. cerevisiae* has uncovered a large number of mutants (*vps*) that missort and secrete vacuolar hydrolases [84], [85], [86]. A small subset of *vps* mutants exhibit a temperature-conditional growth phenotype and show a severe defect in the localization of soluble vacuolar proteins, yet maintain a near-normal vacuole structure. One gene affected in one of these mutants is *VPS45* which has been found to encode a member of the SM protein family, Vps45p [33].

VPS45 encodes a 67 kDa protein which is involved in delivery of proteins to the yeast vacuole, specifically it functions in a Golgi-to-endosome delivery event [33].

VPS45 deletion mutants missort multiple vacuolar hydrolases, are temperature sensitive for growth, and exhibit class D vacuole morphology [33]. Class D *vps* mutants display wild type morphology, but have a large, single vacuolar structure and exhibit defects in mother-to-daughter cell vacuolar inheritance [33]. The accumulation of 40 – 50 nm vesicles in *VPS45* deletion mutants indicates that like other members of the SM protein family, Vps45p functions at a vesicle docking and fusion event [33].

The peripheral association of Vps45p with cellular membranes and the observation that this association can be saturated suggest that Vps45p may be interacting with a specific and limiting membrane component. A reasonable candidate for this membrane component would be a member of the syntaxin protein family [33].

1.4.2.3. Interactions between Tlg2p and Vps45p

The principal binding site for Vps45p on intracellular membranes is provided by Tlg2p, and Vps45p is required for stable expression of Tlg2p [73].

In 1999, Coe and colleagues first published that Vps45p may regulate the SNARE complex formed by Tlg2p, Tlg1p, Vti1p and Snc2p [75].

Bryant and James (2001) have shown that the deletion of *VPS45* resulted in the cellular depletion of Tlg2p through rapid proteosomal degradation [74]. These data suggested that a major function of SM proteins is to act as chaperone-like molecules for their cognate t-SNAREs and that the levels of the t-SNARE in the cell are closely regulated by the relative expression of cognate SM proteins [74].

Tlg2p binds to Vps45p via a short N-terminal peptide peptide sequence [52] (Mode 2 binding, see 1.2.1.1.).

It was suggested that Tlg2p is incapable of making the transition to the open conformation, which would allow it to interact with its SNARE partners, in the absence of Vps45p [74]. An N-terminal truncation mutant of Tlg2p bypasses the requirement for SNARE complex formation on Vps45p since it cannot adopt the closed conformation, and consequently permanently mimics the open conformation with its core-binding domain available to bind Tlg1p and Vti1p [74]. These data also suggest that SM protein play an active role in the conformational switch from the closed to open state thus activating the t-SNARE and facilitating membrane fusion [74].

Further analysis with a range of Tlg2p fragments showed that the first 33 N-terminal residues of Tlg2p are sufficient for Vps45p binding [52].

It was demonstrated that Vps45p binds to Tlg2p in its monomeric state before the formation of the trans-SNARE complex, then it dissociates and is released into the cytosol. The conversion of trans-SNARE complex to the cis-configuration defines membrane fusion and in this exact time Vps45p reenters the cycle by binding to the cis-complex. The cycle is completed after disassembly of cis-SNARE complexes with Vps45p bound by Sec18p (ATPase NSF, see 1.3.) presumably resulting in the Vps45p-Tlg2p complex that is then ready to receive incoming vesicles in the process of docking [87].

Carpp and colleagues disrupted the binding of Tlg2p to Vps45p via its N-terminal region and demonstrated that membrane traffic is not perturbed. They also reported a dominant-negative version of Vps45p, which has revealed that the SM protein interacts with the Tlg2p–SNARE complex through a mechanism distinct from the Sed5p–Sly1p hydrophobic pocket mode of binding [41] (Mode 3 binding, see 1.2.1.1.).

It was later discovered that selective disruption of the pocket-mode of binding between Vps45p and Tlg2p results in decreased cellular levels of the syntaxin and that stabilization of Tlg2p alone is not sufficient to restore the trafficking defects that result from loss of Vps45p [88].

Recently a second binding site for Vps45p on Tlg2p was discovered which corresponds to a Tlg2p closed conformation [89]. Furthermore it was shown that the N-terminal peptide of Tlg2p modulates the affinity of the closed conformation binding site, indicating a role for the N-peptide in controlling accessibility of Tlg2p for SNARE complex assembly [89]. These data suggest that a common mechanism for SM-syntaxin interactions is a dual mode, whereby the SM interacts with two distinct sites on the syntaxin, the N-terminal peptide and the closed conformation [89].

1.5. Homology between syntaxin 16and Tlg2p

Sequence analyses identified the yeast syntaxin Tlg2p as the closest structural *S. cerevisiae* homologue of the TGN- localized mammalian syntaxin 16 [50].

Syntaxin 16 was later also described as a functional homologue of Tlg2p. Its expression complements many of the trafficking phenotypes displayed by yeast cells lacking Tlg2p [90].

Similarly, the other SNARE complex binding partners of syntaxin 16 show homology to the Tlg2p complex binding partners. The coiled-coil domain of Tlg1p shows homology to mammalian syntaxin 6 (28% identity and 58% similarity) [75], Vti1p from *S. cerevisiae* shows similarity with mammalian Vti1a [76] and Vps45p is homologous to mVps45p.

The Tlg2p/ Vps45p binding mode is also conserved in the mammalian syntaxin 16 and mVps45p, supporting the notion that these are true homologs of the yeast Tlg2p and Vps45p. Tlg2p and syntaxin 16 share the N-terminal peptide motif that is responsible for binding to Vps45p [52] (Fig. 5).

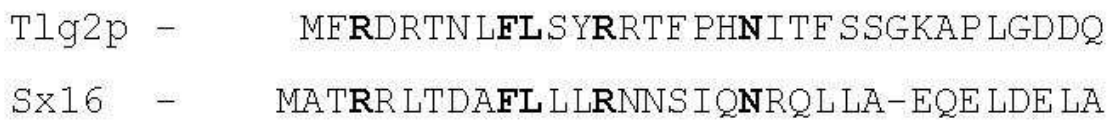

Figure 5: Sequence alignments of the first 33 amino acids of Tlg2p and syntaxin 16. Those residues conserved between Tlg2p and syntaxin 16 (Sx16) are shown in bold. (modified from [52]).

Also the cross-reactivity of mammalian syntaxin 16 and mVps45 with the yeast Vps45p and Tlg2p, respectively also reinforces the notion that they are true homologs of these proteins [52].

1.6. Trafficking of carboxypeptidase Y

Transport of carboxypeptidase Y and it's missorting during the vesicle transport is a tool that can be used to explore the endocytic transport pathway in yeast [91], [92]. Maturation and localization of the yeast vacuolar carboxypeptidase Y requires at least two organelles and 12 cellular functions that were previously identified as components of the

secretory apparatus [92]. The vacuolar glycoprotein carboxypeptidase Y (61 kDa) is biosynthesized in ER on ER-bound ribosomes as an inactive precursor (pro carboxypeptidase Y or p1 carboxypeptidase Y, 67 kDa) [92]. The inactive precursor (hydrolase) exists only during the intracellular transport to the vacuole, where the pro-enzyme is processed to the mature active enzyme [93]. The conversion *in vivo* of the pro-enzyme into the mature form has a halflife of 6 minutes [93]. In the Golgi system, p1 carboxypeptidasde Y is modified further by glycosylation into the form p2 carboxypeptidase Y that has size of 69 kDa [92]. SNARE proteins are required for the transport and therefore also important for the maturation of premature carboxypeptidase Y. Tlg2p, the protein which is studied in this thesis is one of the SNARE proteins involved in transport of carboxypeptidase Y. Cells lacking *TLG2* secrete 15-20% of premature carboxypeptidase Y into the growth medium.

2. Aims

- To find a functional phenotype for yeast Tlg2p SNARE phosphorylation that could be used to analyse Syntaxin 16 phosphorylation by studying:
  - Stability of phosphorylated/dephosphorylated protein Tlg2p in yeast
  - Missorting of Carboxypeptidase Y
  - Salt/Osmotic stress sensitivity
  - Binding of SNARE complexes containing Tlg2p to SM protein Vps45p
- To construct hybrid SNARE complex made from mammalian SNARE protein syntaxin 16 and yeast SNARE proteins Tlg1p, Vti1p and Snc2p
  - To asses binding of hybrid SNARE complex to SM protein Vps45p

3. Materials and Methods

3.1. Materials

3.1.1. Laboratory equipment

Laboratory equipment used in this study is listed in Tab.1.

Table 1 - Laboratory equipment used in this study		
Equipment	Brand	Type
Centrifuge	Beckman Coulter Microfuge 22R	F241.5P Rotor, 14 000 RPM, 17 500 x g
Sonikator	MSE Soniprep	150
Centrifuge	Beckman Coulter Allegra X-12R	Swinging-Bucket rotor, 3750 RPM, 3270 x g
Incubator Shaker	Kühner Switzerland ISF-1-W-Shaker	Climo Shaker
Heatblock	Grant	QBT2
Sterilisator	Prestige Medical	2100 Classic
Spectrophotometer	Jenway Genova	DNA Life Science Analyser
Ultracentrifuge	Beckman J2-21	JA-20
Gel Documentation System	Bio-Rad	Gel Doc XR+ System
Western Blot, SDS-PAGE apparatus	Bio-Rad	
Incubator	Gallenkamp	

3.1.2. Reagents and enzymes

Reagents used in this study are listed in Tab. 2, kits used in this study are in Tab. 3 (page 32), restriction enzymes are in Tab. 4 (page 32) and other enzymes are in Tab. 5 (page33).

Table 2 - Reagents used in this study	
Reagents	Company
Ni-NTA Agarose	Quiagen
IgG Sepharose 6 Fast Flow	GE Healthcare
TEMED	Sigma

DMEM (41965)	Gibco
Ampicilin	Sigma
L-Glutamine (200mM, 100x, 25030)	Gibco
Trypsin 0,05% (25300)	Gibco
DTT	Sigma
EDTA	Reidel-deHaën
Dried Skimmed Milk	Marvel
Sodium chloride	Normapur
Potassium chloride	Normapur
KH2P/NA2HPO4	Normapur
30% Acrylamide/Bisacrylamide (37:5:1 ratio) Stock Solution	Severn Biotech
Nitrocellulose Transfer Membrane	Whatman, Protran
Agarose	Roche
IPTG	Melford
Ethidium Bromide	Gene Choice
SDS	Sigma
Tris	Sigma
Glycerol	Sigma
Bromphenol blue	SERVA
Glycine	Sigma
Tween	Sigma
Methanol	Fisher Scientific
Acetic acid	Fisher Scientific
Coomassie Brilliant blue	Sigma
Trichloracetic acid	Sigma

Table 3 - Kits used in this study	
Reaction Kits	Company
Promega Wizard® Plus SV Miniprep kit	Promega
QIAquick Gel Extraction Kit	QIAGEN

Table 4 - Restriction enzymes used in this study		
Restriction Enzyme	From	Buffer (% activity in Buffer)
NdeI	Promega	Buffer D (100%)
XhoI	Promega	Buffer D (100%)
PstI	New England Biolabs	Buffer 2 (75%), 3 (100%), 4 (50%)
HindIII	New England Biolabs	Buffer 2 (100%)
MscI	New England Biolabs	Buffer 3 (75%), 4 (100%)
NcoI	New England Biolabs	Buffer 3 (100%)

Table 5 - Other enzymes used in this study		
Enzyme	Company	Buffer
T4 DNA Ligase	New England Biolabs	Ligase Buffer

1 kb ladder and 6 x loading dye used in this study were obtained from either Promega (Southampton, UK) or New England Biolabs (Hitchin, UK).

3.1.3. Bacteria and yeast strains

The bacteria (*E. coli*) and yeast (*S. cerevisiae*) strains used in this study are listed in Table 6.

Table 6 - *E. coli* and *S. cerevisiae* strains used in this study		
E. coli strains used in this study:		
Strain	Genotype	Source
BL-21 Star™ (DE3)	F- *omp*T *hsd*SB(rB-mB-) *gal dcm rne131* (DE3)	Invitrogen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔM15 Tn*10 (Tetr)*].	Stratagene
SCS110	*rpsL (Strr) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lac-proAB)* [F' *traD36 proAB lacI*^{q}ZΔM15]	Stratagene
S. cerevisiae strains used in this study:		
Strain	Genotype	Reference
RPY10	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2*	[94]
SF838-9D	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2 pep4-3*	[85]
NOzY4	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2 tlg2Δ::Kanr*	[74]
NOzY2	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2 vps45Δ::Kanr*	[74]
NOzY3	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2 pep4-3 tlg2Δ::Kanr*	[74]
NOzY1	MATα *ura3-52 leu2-3, 112 his4-519 ade6 gal2 pep4-3 vps45Δ::Kanr*	[74]

3.1.4. Growth media

Reagents used in this study for yeast and *E. coli* growth media are listed in Tab. 7.

Table 7 - Reagents for Yeast and *E. coli* growth media used in this study	
Yeast and *E. Coli* media reagents	Company
Yeast Nitrogen Base	Formedium
Synthetic defined medium: -ura-met	Formedium
Mico agar	Formedium
Yeast extract	Formedium
Peptone	Formedium
Tryptone	Formedium
Terrific Broth	Melford

Bacterial cells were grown in:

- 2xYT (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl).
- Solid medium was generated by the addition of 2% (w/v) micro agar.
- Plasmid selection was achieved by adding the antibiotic ampicillin to the medium at a final concentration of 100 μg/ml or chloramphenicol at a final concentration of 34 μg/ml.
- Terrific Broth medium was used for culturing bacteria cells for protein purification

Yeast cells were grown in:

- YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose).
- Solid medium was generated by the addition of 2% (w/v) micro agar.
- Plasmid selection was achieved using minimal synthetic defined (selective) medium, synthetic defined medium (0.675% (w/v) yeast nitrogen base without amino acid, 2% (w/v) glucose) lacking uracil and methionin (add 0.185% (w/v) synthetic defined medium: -ura-met)
- Solid medium was generated by the addition of 2% (w/v) micro agar.

3.1.5. Antibodies

3.1.5.1. Primary antibodies

Primary antibodies used in this study are listed in table 8.

Table 8 - Antibodies used in this study			
Primary Ab	Dilution	Diluting Buffer	From
αTlg2p	1:500	PBST	rabbit
αPgk1p	1:10000	PBST	rabbit
αHA	1:1000	PBST	rat
αCPY	1:300	TBST	mouse
αVti1p	1:200	PBST	rabbit
αSnc2p	1:200	PBST	rabbit
αTlg1p	1:5000	PBST	rabbit
αVps45p	1:600	PBST	rabbit

CPY – carboxypeptidase Y

αTlg2p - Polyclonal antibodies against Tlg2p were raised in rabbits immunized with peptides corresponding to residues 272-287 and 381-396 (this was performed by Eurogentec). Antibodies present in the anti-serum were affinity purified using PrA-agarose (see Section 2.2.11.1; ~0.4 mg/ml) and were generally used at a 1:100 dilution in immunoblot analysis.

αPgk1 - Rabbit polyclonal anti-Pgk1p anti-serum has previously been described in [94] and was used at a 1:20000 dilution.

αHA - Monoclonal antibodies raised in rats (Clone 3F10) IgG1, recognizing influenza viral protein hemagglutinin (HA) peptide sequence YPYDVPDYA. Purchased from Roche Diagnostics Ltd (catalogue # 11815016001). Used at a 1:3000 dilution

α carboxypeptidase Y - Mouse monoclonal anti-carboxypeptidase Y antibodies (clone 10A5; 20 μg/ml) have previously been described in (Roeder and Shaw, 1996) and were used at a 1:50 dilution for immunoblot analysis.

αVti1p - Rabbit polyclonal anti-Vti1p anti-serum has previously been described in (Coe et al., 1999) and was used at a 1:1000 dilution.

αSnc2p - Polyclonal antibodies against Snc2p were raised in rabbits immunized with peptides corresponding to residues 11-25 and 72-86 (this was performed by Eurogentec). Antibodies specific to residues 11-25 were affinity purified from the rabbit anti-serum by Eurogentec. Purified anti-Snc2p antibodies (~0.7 mg/ml) were used at a 1:1000 dilution for immunoblot analysis.

αVps45p - Polyclonal antibodies against Vps45p were raised in rabbits immunized with peptides corresponding to residues 14-28 and 563-577 (this was performed by Eurogentec). Antibodies specific to residues 563-577 of Vps45p were affinity purified from the rabbit anti-serum using a SulfoLink Kit (Pierce Biotechnology, cat # 44895; see Section 2.2.11.2). Purified anti-Vps45p antibodies (~0.6 mg/ml) were used at a 1:500-1:1000 dilution for immunoblot analysis.

3.1.5.2. Secondary antibodies

All secondary antibodies used in immunoblot analysis were conjugated to horseradish peroxidase (HRP) and were purchased from GE Healthcare (Buckinghamshire, UK).

Anti-rabbit IgG, HRP-linked antibodies (cat # NA934)
Anti-mouse IgG, HRP-linked antibodies (cat # NA931)
Anti-rat IgG, HRP-linked antibodies (cat # 5546)
All these antibodies were used at a dilution of 1:1000.

3.1.6. Plasmids

Plasmids used in this study are listed in table 9.

Table 9 - Plasmids used in this study

No. of plasmid	Description
Yeast expression plasmids:	
#284	pVT102u
#50	pVT102u - HA.TLG2 (WT) [65]
#85	pVT102u - HA.TLG2 (S90A) (in our laboratory)
#86	pVT102u - HA.TLG2 (S90D) (in our laboratory)
#348	pVT102u - HA.STX16 (WT) [90]

E. Coli expression plasmids:	
#12	pACY-DUET (Novagen)
#112	pET-Duet-PrA (Novagen)
#113	pET-Duet - WT Tlg2cyto-PrA + Vti1cyto [41]
#154	pACY-DUET - Tlg1cyto + His-Snc2cyto [41]
#228	pET-Duet - S90A Tlg2cyto-PrA + Vti1cyto (in our laboratory)
#229	pET-Duet - S90D Tlg2cyto-PrA + Vti1cyto (in our laboratory)
BV001	pET-Duet – syntaxin16-cyto + Vti1cyto (this study)

3.2. Methods

3.2.1. Purification of plasmid DNA from bacteria

Five ml of 2xYT medium containing ampicilin (final concentration 100μg/ml, filter sterilized) was inoculated by the *E. coli* strain containing the desired plasmid, and grown over night at 37^{o}C with constant shaking. Cells were harvested by centrifugation at 3610 g for 5 min. The plasmid DNA was isolated by using Promega Wizard® Plus SV Miniprep kit (Promega, Southampton, UK). Plasmid DNA was stored at -20°C.

3.2.2. Competent yeast cells preparation

The culture was grown in 100 ml YPD medium with constant shaking until they reached mid-log phase (OD_{600} of 0.5-1.0). Cells were harvested by centrifugation at 3610 g for 3 min. Pellets were resuspended in 10 ml of LiTE-Sorb, then repelleted (3610 g, 3 min) and again resuspended in 1 ml of LiTE-Sorb. Resuspended cells were incubated at 30°C with constant shaking for 1 hour. For storage was added an equivalent volume of ice cold 40% glycerol + 0.5% NaCl. Then cells were incubated on ice for around 20 min. Aliquots were stored at -80°C.

LiTE-Sorb:

0.1M LiOAc

10mM Tris.HCl (pH 7.6)

1mM EDTA

1.2M sorbitol

3.2.3. Yeast cells transformation

Competent yeast cells were transformed as follows. Into 1.5ml micro tube tube was added 100µl of competent yeast cells, 10µl of DNA and 120µl of 70% (w/v) PEG-3350 and the content of the tube carefully mixed by inversion to ensure proper mixing (not vortexed as this can cause damage to competent cells). The mixture was incubated with constant shaking at 30°C for 30 min. After the incubation, cells were heat-shocked at 42°C for 20 min and then harvested by centrifugation at 1430 g for 2 min. Pellets were resuspended in 200µl of sterile dH_2O. Resuspended cells were plated onto selective synthetic defined medium-ura-met containing 2% (w/v) micro agar and incubated for 2-4 days at 30°C.

3.2.4. Preparation of yeast whole cell lysate for electrophoresis and immunoblot analysis

A colony of transformed yeast was picked from a fresh selective plate and inoculated into 10 ml of selective synthetic defined medium-ura-met. The culture was grown overnight with constant shaking at 30°C. To assess the steady-state level of protein in yeast whole cell lysates, a volume of culture equivalent to 10 OD_{600} units was harvested by centrifugation at 3610 g for 3 minutes, then transferred to 1.5ml micro tubes. The supernatant was carefully removed by aspiration. The cell pellets were then resuspended by the addition of 100µl of Twirl buffer + 10% (v/v) β-mercaptoethanol. Samples were vortexed and put into the dry heat block for 10 minutes at 65°C. Before using for SDS-PAGE electrophoresis, samples were briefly vortexed and stored on ice.

Twirl buffer:

50mM Tris-HCl (pH6.8)

5% (w/v) SDS

8M Urea

10% (v/v) Glycerol

0.2% Bromphenol Blue

3.2.5. SDS-PAGE electrophoresis

Proteins were separated by using discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). For preparing gels was used Bio-Rad Mini-Protean apparatus. Bottom separating gel was prepared as follows. For analysis in this study usually 10% (v/v) acrylamide gels were used (occasionally 15% SDS-PAGE gels were used depending on the molecular weight of the protein to be analysed).

10% Acrylamide gel:

10% (v/v) acrylamide-bisacrylamide mixture (37.5:1 ratio)

0.75M Tris-HCl (pH 8.8)

0.2% (w/v) SDS

15% Acrylamide gel:

15% (v/v) acrylamide-bisacrylamide mixture (37.5:1 ratio)

0.75M Tris-HCl (pH 8.8)

0.2% (w/v) SDS

As top stacking gel was used 5% Acrylamide gel prepared as follows.

5% Acrylamide gel:

5% (v/v) acrylamide-bisacrylamide mixture (37.5:1 ratio)

0,25M Tris-HCl (pH 6.8)

0.2% (w/v) SDS

As samples for electrophoresis in this study, we used either whole cell lysate Twirl samples (see 3.2.4.) or samples prepared in 2x LSB + 10% (v/v) β-mercaptoethanol. Purified protein samples were resuspended in the same volume of 2x LSB + 10% (v/v) β-mercaptoethanol. Proteins were denaturated on dry heat block at 95°C for 5 minutes. Samples to be ran on 15% acrylamide gels were generally prepared by resuspending in 1x LSB + 10% (v/v) β-mercaptoethanol denaturated also on dry heat block at 95°C for 5 minutes.

2x Laemmli Sample Buffer:

100mM Tris-HCl (pH 6.8)

4% SDS

20% Glycerol

0.2% bromphenol blue

3.2.6. Western blot and immunoanalysis

In this study, a semi-dry transfer apparatus (Bio-Rad Trans-Blot® SD cell) was used in order to transfer proteins from the polyacrylamide gel to the nitrocellulose membrane. A piece of nitrocellulose membrane (Whatman Protran, 0.45 μ pore size) slightly larger than the size of gel (10 x 7 cm gels were used) and 6 pieces of Whatman 3MM paper were used. The paper and membrane were all pre-soaked in semi-dry transfer buffer. Into the apparatus were placed 3 layers of pre-soaked Whatman 3MM paper, then the nitrocellulose membrane. On the membrane was put polyacrylamide gel containing samples separated by electrophoresis, then another 3 layers of pre-soaked Whatman 3MM paper. All air bubbles were carefully removed to prevent bad transfer. The semi-dry transfer cell was set on constant current 0.18A for 45 minutes (for 2 membranes).

After the transfer, membranes were blocked in 5% (w/v) non-fat milk powder resuspended in PBST or TBST according to antibody used (see Table 10).

Membranes were put into primary antibody according to what was studied. Primary antibody was diluted in 1% (w/v) non-fat milk resuspended in either PBST buffer or TBST buffer. After at least 2 hours, but usually over-night, membranes were washed 6 times for 5 min in either PBST, or TBST and put into the secondary antibody based on the primary antibody. Secondary antibody was diluted in 5% (w/v) non-fat milk resuspended in either PBST or TBST. After one hour, membranes were again washed 6 times for 5 min in PBST or TBST and developed using ECL solution.

Table 10 - Antibodies used in this study -Dilution

Primary Ab	Dilution	Diluting Buffer	Secondary Ab
αTlg2p	1:500	PBST	rabbit
αPgk1p	1:10000	PBST	rabbit
αHA	1:1000	PBST	rat
αCPY	1:300	TBST	mouse
αVti1p	1:200	PBST	rabbit
αSnc2p	1:200	PBST	rabbit
αTlg1p	1:5000	PBST	rabbit
αVps45p	1:600	PBST	rabbit

CPY – carboxypeptidase Y

3.2.7. Coomassie visualisation of proteins

Protein separation was visualised by incubating the gel in Coomassie Brilliant Blue solution constantly shaking for 1 hour followed by destaining in destain solution until the bands were clearly visible (at least 30 mins).

Coomassie Brilliant Blue solution:

0.25 g Coomassie Brilliant Blue R250

In methanol:dH_2O:glacial acetic acid (4.5:4.5:1 v/v/v)

Destain solution:

5% (v/v) methanol

10% (v/v) glacial acetic acid

3.2.8. Trichloracetic acid precipitation of secreted carboxypeptidase Y

Ten ml of selective synthetic defined medium-ura-met was inoculated by yeast strain transformed with the appropriate plasmid and left constantly shaking in the incubator at 30°C over night. Cultures were diluted into OD_{600} 0.2 in 20 ml of new selective synthetic defined medium-ura-met. Yeast cells were left to grow under constant shaking at 30°C in the incubator until they reached OD_{600} 0.8. Cells were harvested by centrifugation at 3610 g for 5 min. Eighteen ml of supernatant was collected and put into new conical tubes. Then trichloracetic acid (TCA) was added to the final concentration of 10 % and left incubated on ice over night. The precipitated proteins were collected by centrifugation at 16160 g for 10 min at 4°C. The resultant pellets were washed by ice cold acetone and repelleted by centrifugation at 16160 g for 5 min at 4°C. This was repeated twice, the supernatant was then drained out before letting the pellets to dry for a couple minutes at low tempered dry heat block. The precipitated proteins were finally resuspended in LSB (500µM Tris-HCl pH 6.8, 10% (v/v) glycerol, 5% (w/v) SDS, 0.2% (w/v) Bromphenol blue, 10% (v/v) β-mercaptoethanol) and vortexed. 1µl of saturated Tris-HCl (pH 8.0) was added to the samples that had turned yellow in colouration (result of low pH). The precipitated proteins were separated using SDS-PAGE and the amount of carboxypeptidase Y secreted assessed using immunoblot analysis with an antibody specific to carboxypeptidase Y protein.

3.2.9. Test for salt/osmotic stress sensitivity of yeast

Yeast cells transformed with appropriate plasmid were grown over night in selective synthetic defined medium-ura-met at 30°C. Amount of cells equivalent to 10 OD_{600} units was harvested by centrifugation at 3610 g for 3 min. The resultant cell pellets were resuspended in 1 ml of sterile dH_2O. Subsequent dilutions of this culture were performed generating cultures with a final concentrations of 10 OD_{600}/ml, 1 OD_{600}/ml, 0.1OD_{600}/ml and 0.01 OD_{600}/ml. Five µl of each culture was spotted onto appropriate medium: synthetic defined medium-ura-met, synthetic defined medium-ura-met + 100mM LiCl, synthetic defined medium-ura-met + 1.75M KCl, synthetic defined medium-ura-met + 1.5M NaCl. Colonies on plates were grown at 30°C for 2-3 days before analysis.

3.2.10. Preparation of competent *E.coli* cells and bacterial transformation

Competent *E. coli* cells were prepared as follows. Five ml of rich medium 2xYT was inoculated by *E. coli* and left to grow overnight. This culture was used to inoculate 500 ml of the same rich 2xYT medium. Cells were left to grow at 37°C with constant shaking in incubator until they reached mid-log phase (an optical density at 600 nm OD_{600} of 0.4 – 0.6). Cells were harvested using centrifugation at 2095g for 10 min at 4°C and then the cell pellet was resuspended in 50 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for one hour. Cells were repelleted by using centrifugation at 2095 g for 10 min at 4°C. Resulting pellet was resuspended in 20 ml of ice-cold storage buffer (0.1 M $CaCl_2$/15% glycerol). Competent cells were divided into 50 µl aliquots and stored at -80°C.

Competent *E. coli* cells were transformed as follows. Appropriate amount of competent cells (according the type, Tab. 11, page 43) was mixed in 1.5 ml micro tube tubes with 2 µl of plasmid DNA. Tubes were inverted to ensure proper mixing, and left on ice for 30 min. For the transformation of cells heat shock method was used. Cells were heat-shocked for 45 sec at 42°C and then again left on ice for 1 min. 950 µl of 2xYT medium was then added and cells were allowed to recover for 1 hour at 37°C with constant shaking.

Table 11 - amount of competent *E. coli* cells used for transformation	
Type of cells	Amount used
One Shot®BL21 Star (DE3), invitrogen	50 µl
XL-1 Blue (generated in our lab)	100 µl
SCS110 (generated in our lab)	100 µl

The cells were harvested by centrifugation at 16160 g for 5 min and the resultant pellets were resuspended in 150 µl of 2xYT and plated onto 2xYT containing ampicillin (100 µg/ml) and incubated overnight at 37°C or entire transformation was used for inoculate 5 ml of 2xYT medium containing appropriate antibiotics (in this study ampicllin – 100 µg/ml and chloramphenicol – 34 µg/ml were used) and used for next assay.

3.2.11. Recombinant protein expression and purification – Preparation of *in vitro* SNARE complexes

Chemically competent *E. Coli* cells BL21 Star™ (DE3) (Invitrogen) were transformed with plasmids driving the expression of proteins for making *in vitro* SNARE complexes (Table 12) as is shown in Table 13.

Table 12 - Plasmids used for transformation for making *in vitro* SNARE complexes	
No. of plasmid	Plasmid
# 12	pACY-DUET
# 112	pET-Duet-PrA
# 113	pET-Duet - WT Tlg2cyto-PrA + Vti1cyto
# 154	pACY-DUET - Tlg1cyto + His-Snc2cyto
# 228	pET-Duet - SA Tlg2cyto-PrA + Vti1cyto
# 229	pET-Duet - SD Tlg2cyto-PrA + Vti1cyto

Table 13 - *E. coli* transformations for making *in vitro* SNARE complexes		
No. of reaction	Plasmid 1 + amount	Plasmid 2 + amount
1	# 113 - 2 µl	# 154 - 2 µl
2	# 228 - 2 µl	# 154 - 2 µl
3	# 229 - 2 µl	# 154 - 2 µl
4	# 112 - 2 µl	# 12 - 2 µl
5	no	no
6	no	no

Five ml of 2xYT medium with appropriate antibiotics was inoculated by the entire transformation. Into the reactions 1-4 ampicilin (100 μg/ml) and chloramphenicol (34 μg/ml) were added. Into the reaction 5 was added just ampicilin (100 μg/ml) and into the reaction 6 just chloramphenicol (34 μg/ml). Reactions number 5 and 6 were used as controls for the transformation. Cells were grown overnight at 37°C with constant shaking. Overnight starter cultures were used to inoculate 500 ml of Terrific broth medium (with added 8 ml of 50 % glycerol and autoclaved) containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Cells were grown at 37°C with constant rotation until they reached mid-log phase (OD_{600} 0.5-0.7). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1mM was added for induction of protein expression and cells were left to grow for 4 hours at constant rotation at 37°C. Cells were harvested using centrifugation at 3273g for 20 mins. Cell pellets were resuspended in 25 ml of PBS by vortexing and repelleted again at 3610 g for 10 minutes. Cell pellets were frozen and stored at -20°C. Frozen cell pellets were thawed on ice and diluted with 25 ml of PBS. Lysis was then performed by incubating the cells on ice in the presence of lysozyme at a final concentration of 1 mg/ml for 30 mins, followed by 6 times 20-sec burst of sonication (Sanyo soniprep 150) with intervals of 20 secs rests on ice. Lysed cells were clarified by centrifugation at 48400 g for 25 mins at 4°C.

3.2.11.1. Purification of PrA tagged proteins

For binding of PrA tagged proteins was used IgG Sepharose™ 6 fast flow (GE Healthcare). 500 μl of settled IgG Sepharose was pre-equilibrated by washing in 2 ml of TST followed by 1 ml of HAc pH 3.4 (0.5M acetic acid adjusted to pH 3.4 with ammonium acetate) then again 1 ml of TST, 1 ml of HAc pH 3.4 and 1 ml TST.

TST (Tris-saline Tween 20):

50 mM Tris-HCl pH 7.6

150 mM NaCl

0.05% Tween 20

Pre-equilibrated IgG Sepharose was incubated with cleared lysate prepared as described above in 3.2.11. with rotation for 2 hours at 4°C. After the incubation the mixture was spun down at 70 g for 2 min to remove the unbound proteins and pelleted IgG Sepharose with bound proteins was washed 8 times with 1 ml of TST (between washes spun down by centrifugation at 70 g for 1 min). The supernatant was removed and for cleavage of the proteins from IgG Sepharose were added 500 μl of PBS and 25 U of Thrombin. All was left to

rotate at room temperature for 4 hours. The cleaved proteins in the supernatant were transferred into another 1.5 ml micro tube and used for next purification step described below in 3.2.11.2.

3.2.11.2. Purification of His tagged proteins

Five hundred µl of settled Ni-NTA Agarose (Qiagen) was washed 5 times with 1 ml of PBS + 20mM Imidazole. Supernatant containing thrombin-cleaved proteins that were prepared as described in section 3.2.11.1. was added to the washed Ni-NTA agarose and incubated at 4°C with rolling for 1 hour. After incubation, the Ni-NTA agarose beads were washed 4 times with 1 ml of PBS + 20mM Imidazole.

Twenty µl of settled Ni-NTA agarose resin with bound proteins was transferred into a new 1.5 ml micro tube. An equivalent volume of 2xLSB + 10% β-mercaptoethanol (20 µl) was then added prior to incubation at 95°C for 5 mins. The Ni-NTA Agarose was then pelleted by centrifugation at 70 g for 1 min and the proteins present in the eluate separated by SDS-Page. The amount of proteins was assessed by Coomassie staining of the gel.

Rest of the Ni-NTA agarose was used for binding of Vps45p from yeast lysate using a pull-down assay as described in section 3.2.12.

3.2.12. Vps45p pull-downs from yeast lysate

Yeast cells transformed by appropriate plasmid (containing HA tagged Vps45 protein) were grown at constant shaking in 10 ml of selective synthetic defined medium-ura-met overnight. Cell culture was diluted into new 10 ml of selective synthetic defined medium-ura-met at OD_{600} 0.2. Cells were grown until they reached mid-log phase (OD_{600} 0.7-1.0). Cells were harvested by centrifugation at 931 g for 2 min. Resulting pellets were resuspended in 1 ml of binding buffer.

Binding buffer:

40 mM HEPES.KOH pH 7.4

150 mM KCl

1mM EDTA

0.5% NP-40

Cells were repelleted again at 931 g for 2 mins and again resuspended in binding buffer in 100 µl per 1 OD_{600}. Amount of glass beads equivalent to half the volume of

resuspended cells was added and the cells lysed by vortexing 4 times for 30 sec at 4°C each time with 1 min interval on ice. Unlysed cells and the glass beads were removed by centrifugation at 70 g for 5 mins. A sample of lysate was saved for later analysis. Lysates were then added to Ni-NTA Agarose prebound with purified *in vitro* SNARE complexes containing Tlg2cyto(WT)-PrA, (or Tlg2cyto-S90A-PrA or Tlg2cyto-S90D-PrA), plus Tlg1cyto, Vti1cyto and Snc2-His tagged (see section 3.2.11.) and rotated overnight in 4°C. To equalise the amount of protein (*in vitro* SNARE complexes) bound on Ni-NTA agarose beads used in this assay, Coomassie stained gels were prepared as described in section 3.2.11.2., and the volume of beads and proteins were then adjusted accordingly. A sample of lysate after binding was saved for later analysis. Ni-NTA agarose was washed 5 times with 1 ml of PBS + 20mM Imidazole. Ni-NTA Agarose resins were settled by centrifugation at 931 g for 2 mins. Supernatant was removed and to the resins was added 30 μl of 2xLSB + 10% β-mercaptoethanol (20 μl) prior to incubation at 95°C for 5 mins. Samples were separated using SDS-PAGE electrophoresis and visualised by using immunoblot with anti-HA antibody.

3.2.13. Cloning

3.2.13.1. Restriction

Control restrictions were prepared as follows.

Restriction recipe:

5 μl of DNA

2 μl of appropriate restriction buffer (see tab 3.1.1)

1 μl of restriction Enzyme 1

1 μl of restriction Enzyme 2

2 μl of 10x BSA (if it is necessary for enzymes, if not H_2O was used instead)

9 – 11 μl H_2O

20 μl of mixture

Restrictions for molecular cloning were prepared as follows.

Restriction recipe:

20 μl of DNA

4 μl of appropriate restriction buffer

2 μl of restriction Enzyme 1

2 μl of restriction Enzyme 2

4 μl of 10x BSA (if it is necessary for enzymes, if not $H_2O$was used instead)

8 – 12 μl H_2O

40 μl of mixture

The mixtures were put into the 37°C dry heat block for 2 – 4 hours (depending on activity of enzymes in buffer that was used). Restriction was checked by Agarose electrophoresis as described in section 3.2.13.2.

3.2.13.2. Agarose gels

Samples of DNA were mixed with 2 – 3 μl (according to the amount of sample) of 6x Loading dye (Promega) and were routinely resolved by electrophoresis through 0.8% (w/v) agarose gel containing ethidium bromide (Gene choice, Maryland, USA) in 1x Tris-acetate (TAE) buffer.

Tris-acetate (TAE) buffer:

0.04 M Tris-acetate

0.001 M EDTA

3.2.13.3. Gel purification

Gel extraction was performed using the QIAquick Gel Extraction Kit from QIAGEN (Hilden, Germany). The microcentrifuge extraction protocol was used in this study.

3.2.13.4. Ligation

Ligation recipe:

1 μl of 10x ligase buffer

1 μl of T4 DNA ligase

2 μl vector DNA

6 μl insert DNA

10 μl of mixture

The mixture was left for 2 hours at room temperature, or overnight at 4°C and then, 5 μl of the mixture was transformed into competant *E. coli* cells. As a negative control for ligation a sample containing only the vector DNA, without the insert DNA (water was added to make up the volume) was used.

3.2.13.5. DNA sequencing

DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequence.

4. Results

It was shown that abolishment of putative phosphorylation site of Tlg2p Ser90 (serine 90) leads to dominant effects on the exocytic and endocytic pathways [71].

Therefore, SNARE phosphorylation may regulate membrane fusion events. However the mechanism of this regulation is not known.

In this study we tried to discover more about the functional phosphorylation site on Ser90 in protein Tlg2p (Fig. 6).

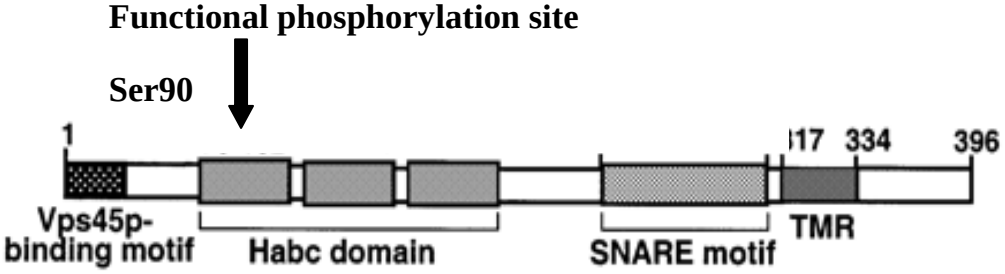

Figure 6: Diagram of structure of gene for Tlg2p with 1 functional phosphorylation site shown on.

We used two different Tlg2p mutants based on this site. First is mutation of serine 90 to alanine (S90A) and second is serine 90 mutated to aspartic acid (S90D). When the serine residue is replaced by alanine, it cannot be phosphorylated, and when the serine is replaced by aspartic acid it mimics phosphorylation of that residue, it is phosphomimetic (aspartic acid group mimics phosphate group). Therefore, we could investigate the effect of phosphorylation or eventually dephosphorylation on Tlg2p function *in vivo* in living yeast organism.

4.1. Stability of Tlg2p mutants S90A and S90D in yeast

4.1.1. Expression of Tlg2p mutants S90A and S90D in *S. cerevisiae* (strain SF838-9D) lacking endogenous Tlg2p (*Δtlg2p* mutant cells)

In order to study the stability of the S90A and S90D mutant versions of Tlg2p compared to wild type Tlg2p in yeast, haemagglutinin (HA)-tagged versions of the proteins

were expressed together in yeast strain NOzY3 (SF838-9D Δ*tlg2*) which lacks *TLG2*. Yeast harbouring empty vector (EV) pVT102u were used as a control and as a control we also added the sample of endogenous Tlg2p expressed in WT cells (strain SF838-9D) (Fig. 7).

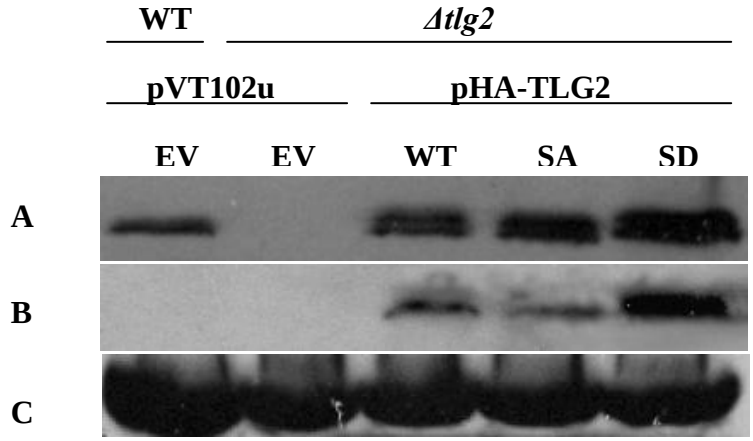

Figure 7: Comparison of Tlg2p phospho-mutant protein levels in SF838-9D and SF838-9D Δ*tlg2* yeast cells.
Wild type yeast cells (strain SF838-9D) were transformed with empty vector pVT102u (EV) and Δ*tlg2* cells (NOzY3; SF838-9Dα *Δtlg2*) transformed with pVT102u (EV), pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D). Cells were grown to mid-log phase in the selective synthetic defined medium-ura-met. Levels of Tlg2p present in whole cell lysates prepared from equal numbers of these transformants (1 OD_{600nm} equivalents) were analysed using SDS-PAGE electrophoresis and Western immunoblot analysis using antibodies as follows: A) anti-serum specific to Tlg2p. B) anti-serum specific to HA. C) anti-serum specific to Pgk1p. Phosphoglucokinase 1 was used as a loading control.
SA – mutant S90A, SD – mutant S90D

All of the pHA-TLG2 constructs were expressed in yeast, but cellular levels of the Tlg2p-S90D mutant in *Δtlg2* cells (strain NOzY3; SF838-9D *Δtlg2*) was significantly higher than the WT and S90A mutant. Since all three versions are expressed from the same promoter, it appears that the higher cellular levels of the Tlg2p-S90D mutant reflect decreased degradation of the protein.

4.1.2. Expression of Tlg2p mutants S90A and S90D in *S. cerevisiae* (strain RPY10) lacking endogenous Tlg2p (*Δtlg2p* mutant cells)

In the previous figure it was shown that cellular levels of HA-Tlg2p WT and both mutants in Δ*tlg2* cells (strain NOzY3; SF838-9D Δ*tlg2*) were not equal, and that S90D mutant expressed Tlg2p at a higher level. In order to equalise cellular levels of the Tlg2p mutants we used yeast strain RPY10 and its congenic Δ*tlg2* mutant (strain NOzY4; RPY10 Δ*tlg2*). These strains are isogenic to SF838-9D and NOzY3 (SF838-9D Δ*tlg2*) respectively except for being *PEP4+* (contains vacuolar protease Pep4p). WT cells (strain RPY10) were transformed with the empty vector pVT102 (EV) and cells lacking Tlg2p (strain NOzY4, RPY10 Δ*tlg2*) were transformed with empty vector pVT102u (EV), pHA-Tlg2p (WT), pHA-Tlg2p (S90A) or HA-Tlg2p (S90D) (Fig. 8).

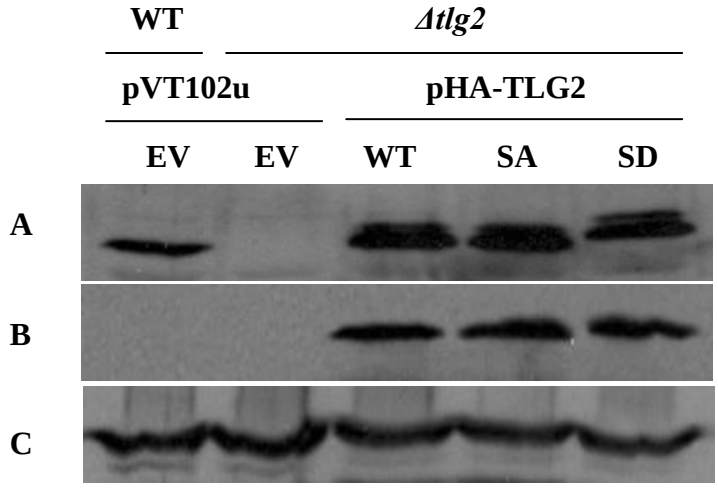

Figure 8: Comparison of Tlg2p mutant protein levels in RPY10 and RPY10 Δ*tlg2* yeast cells.
Wild type yeast cells (strain RPY10) were transformed with empty vector pVT102u (EV) and Δ*tlg2* cells (NOzY4; RPY10 Δ*tlg2*) transformed with pVT102u (EV), pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D). Cells were grown to mid-log phase in the selective synthetic defined medium-ura-met. Levels of Tlg2p present in whole cell lysates prepared from equal numbers of these transformants (1 OD_{600nm} equivalents) were analysed using SDS-PAGE electrophoresis and Western immunoblot analysis using antibodies as follows: A) anti-serum specific to Tlg2p. B) anti-serum specific to HA. C) anti-serum specific to Pgk1p. Phosphoglucokinase 1 was used as a loading control.
SA – mutant S90A, SD – mutant S90D

It can be seen that both mutants and wild type Tlg2p are expressed at a similar levels in RPY10 Δ*tlg2* in contrast to strain NOzY3 (SF838-9D Δ*tlg2*). This indicates that cellular levels of Tlg2p are regulated by the vacuolar protease Pep4p.

4.1.3. Expression of Tlg2p mutants S90A and S90D in *S. cerevisiae* (strain SF838-9Dα) lacking endogenous Vps45p (*Δvps45p* mutant cells)

The point of this experiment was to try to find out if deletion of *VPS45* gene causes differences between expression of Tlg2p-WT, Tlg2p-S90A and Tlg2p-S90D in the yeast strain NOzY2 (SF838-9D Δ*vps45*). Therefore we expressed both mutants S90A and S90D, WT Tlg2p and the empty vector pVT102u (EV) in NOzY2 and as a control we also added the sample of endogenous Tlg2p expressed in WT cells (strain SF838-9D) harbouring an empty vector (Fig. 9).

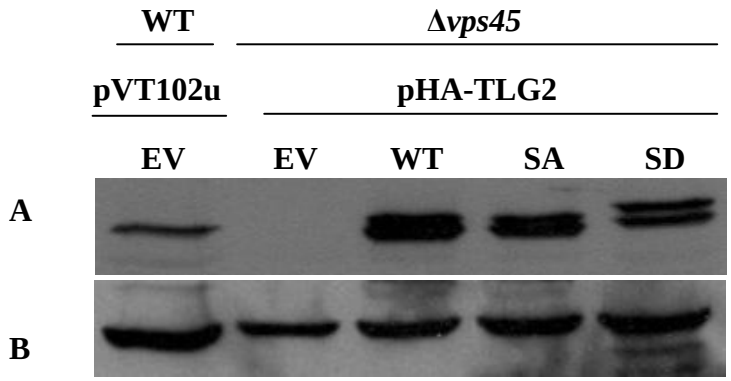

Figure 9: Comparison of Tlg2p cellular levels in WT yeast cells (strain SF838-9D) and NOzY2 yeast strain (SF838-9D *Δvps45*)
Wild type yeast cells (strain SF838-9D) transformed with empty vector pVT102u (EV) and *Δvps45* cells (NOzY2 SF838-9D *Δvps45*) transformed with pVT102u (EV), pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D). Cells were grown to mid-log phase in the selective synthetic defined medium-ura-met. Levels of Tlg2p present in whole cell lysates prepared from equal numbers of these transformants (1 OD_{600nm} equivalents) were analysed using SDS-PAGE electrophoresis and Western immunoblot analysis using antibodies as follows. A) anti-serum specific to Tlg2p. B) anti-serum specific to Pgk1p. Phosphoglucokinase 1 was used as a loading control.
SA – mutant S90A, SD – mutant S90D

It can be seen from our results that there is no significant difference in levels of expression between both mutants (S90A and S90D).

We were also looking at the difference between the expression of wild type and both mutants (S90A, S90D) in *Δtlg2* cells and *Δvps45* (Fig. 10).

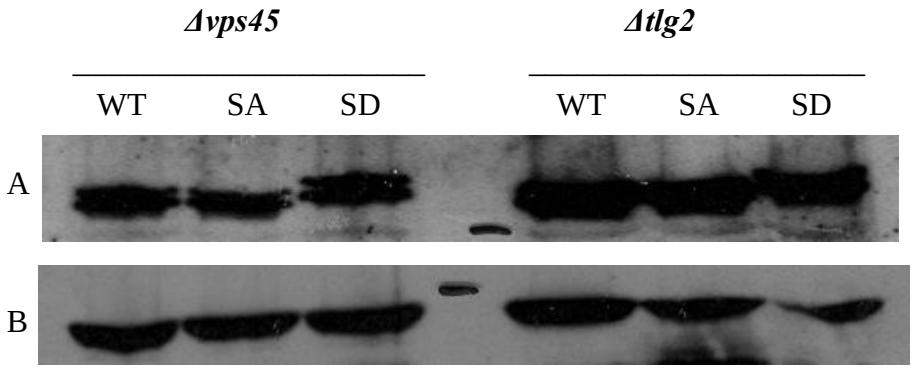

Figure 10: Comparison of expression of Tlg2 (WT), (S90A), (S90D) in NOzY2 and NOzY3 yeast cells
***Δvps45* cells (NOzY2) and *Δtlg2* cells (NOzY3) transformed with pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D) Cells were grown to mid-log phase in the selective synthetic defined medium-ura-met. Levels of Tlg2p present in whole cell lysates prepared from equal numbers of these transformants (1 OD_{600nm} equivalents) were analysed using SDS-PAGE electrophoresis and Western immunoblot analysis using antibodies as follows: A) anti-serum specific to Tlg2p. B) anti-serum specific to Pgk1p. Phosphoglucokinase 1 was used as a loading control.**
SA – mutant S90A, SD – mutant S90D

Despite there is no significant difference between both mutants (S90A and S90D), the important difference is that cellular levels of Tlg2p are higher in *Δtlg2* cells (NOzY3) than in Δ*vps45* cells (NOzY2). This supports the theory that interaction of Tlg2p and Vps45p protect endogenous Tlg2p from degradation.

4.2. Looking for functional phenotype altering in Tlg2p-S90A and Tlg2p-S90D

It was shown before that yeast cells lacking Tlg2p often displays numerous phenotypes as a result of their defective trafficking mechanism through the trans-Golgi network [63], [64], [65]. Some of these defects include: missorting of 15-20% of carboxypeptidase Y, failure to grow on high salt medium and abnormal fragmentation of the

vacuole [63]. In this study, we tried to find some differences between both mutants (S90A, S90D) in the carboxypeptidase Y missorting/secretion phenotype and salt/osmotic stress sensitivity phenotype.

4.2.1. Trichloracetic acid precipitation of missorted/secreted carboxypeptidase Y

Trichloroacetic acid (TCA) was used to precipitate secreted proteins from the growth medium of *Δtlg2* cells (strain NOzY3; SF838-9D *Δtlg2*) expressing HA-Tlg2p(WT), HA-Tlg2p (S90D) and HA-Tlg2p (S90D) or the empty vector pVT102u. As a control WT cells (strain SF838-9D) harbouring empty vector pVT102u were used to look at levels of carboxypeptidase Y secreted with endogenous Tlg2p levels. (Fig. 11).

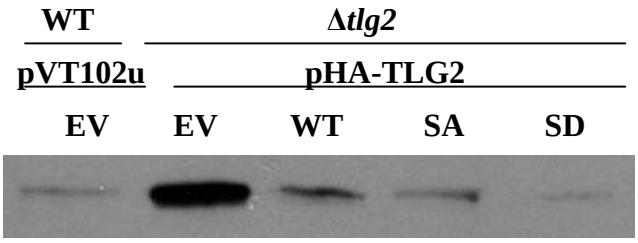

Figure 11: Comparison of missorting/secretion of carboxypeptidase Y in WT yeast cells (strain SF838-9D) and Δ*tlg2* cells NOzY3 yeast strain (SF838-9D *Δtlg2*).
Wild type yeast cells (strain SF838-9D) transformed with pVT102u (EV) and *Δtlg2* cells (NOzY3; SF838-9D *Δtlg2*) transformed with pVT102u (EV), pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D) were grown to mid-log phase in the selective synthetic defined medium-ura-met. Secreted carboxypeptidase Y was then precipitated from growth medium using TCA precipitation. Precipitated protein was resuspended in sample buffer, heated at 95^{o}C for 5 minutes and then analysed by SDS-PAGE electrophoresis and Western blotting using an anti-carboxypeptidase Y antibody.
SA – mutant S90A, SD – mutant S90D

It can be seen in Fig. 11 that all versions of Tlg2p (WT, SA and SD) can complement the carboxypeptidase Y secretion phenotype seen in Δ*tlg2* cells harbouring just the empty vector (EV). It appears that SF838-9D *Δtlg2* cells expressing HA-Tlg2p (S90D) complement better than strains containing HA-Tlg2p (S90A) or HA-Tlg2p (WT). This suggests that the transport of carboxypeptidase Y is affected by the phosphorylation status of Tlg2p. In order to confirm better complementation of S90D mutant we tried to do the same assay but using the

RPY10 yeast strain transformed by pVT102u and RPY10 *Δtlg2* (NOzY4) yeast strain transformed by pVT102u, pHA-TLG2 (WT), pHA-TLG2 (S90A) and pHA-TLG2 (S90D) (Fig. 12).

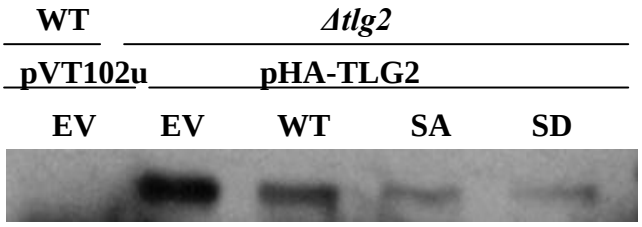

Figure 12: Comparison of missorting/secretion of carboxypeptidase Y in WT yeast cells (strain RPY10) and in *Δtlg2* cells (RPY10 *Δtlg2*)
Wild type yeast cells (strain RPY10) transformed with pVT102u (EV) and *Δtlg2* cells (NOzY4; RPY10 *Δtlg2*) transformed with pVT102u (EV), pHA-TLG2 (WT), pHA-TLG2 (S90A), pHA-TLG2 (S90D) were grown to mid-log phase in the selective synthetic defined medium-ura-met. Secreted carboxypeptidase Y was then precipitated from growth medium using TCA precipitation. Precipitated protein was resuspended in sample buffer, heated at 95 ^{o}C for 5 minutes and then analysed by SDS-PAGE Electrophoresis and Western blotting using an anti-carboxypeptidase Y antibody.
SA – mutant S90A, SD – mutant S90D

According to these results (Fig. 15), there is no significant difference between RPY10 *Δtlg2* (NOzY4) transformed with pHA-TLG2 (S90A) and pHA-TLG2 (S90D). It was shown that missorting of carboxypeptidase Y is not dependent on phosphorylation of Tlg2p at Ser90.

4.2.2. Salt/osmotic stress sensitivity

In this study we investigated if salt sensitivity phenotype is altered by the phosphorylation status of Tlg2p mutants. Cells were grown in selective synthetic defined medium-ura-met before spotting them on to agar plates containing different concentrations of salts to find if and how sensitive Tlg2p phosphorylation mutants are in a high-salt environment compared to wild type cells.

In this experiment we used again wild type RPY10 cells with an empty vector pVT102u and cells lacking *TLG2* (RPY10 *Δtlg2*) with following vectors: pVT102u, pHA-TLG2 (WT), pHA-TLG2 (S90A) and pHA-TLG2 (S90D) (Fig. 13, page 56).

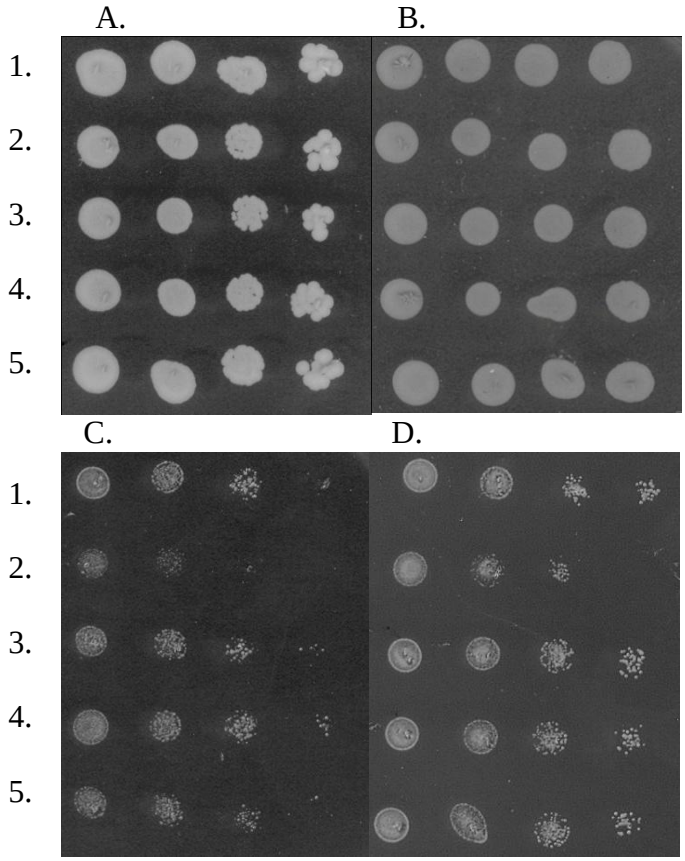

Figure 13: Salt sensitivity/Osmotic stress experiments with Yeast strains containing S90A and S90D mutants of Tlg2p.
Yeast cells transformed with an appropriate plasmid (1. RPY10 + pVT102u, 2. RPY10 *Δtlg2* + pVT102u, 3. RPY10 *Δtlg2* + pHA-TLG2 (WT), 4. RPY10 *Δtlg2* + pHA-TLG2 (S90A), 5. RPY10 *Δtlg2* + pHA-TLG2 (S90D) were grown to the mid-log phase (OD_{600} 0.6-1.0) in the selective synthetic defined medium-ura-met. Amount of cells for 10 OD_{600} was harvested by centrifugation at 3610 g for 3 minutes. Pellets were diluted in 1 ml of sterile H_2O. Then we made a row of serial-dilutions 10 OD/ml, 1 OD/ml, 0.1 OD/ml and 0.01 OD/ml. 5µl of each dilution was spotted onto the appropriate medium.
A. synthetic defined medium-ura-met, B. synthetic defined medium-ura-met + 100mM LiCl, C. synthetic defined medium-ura-met + 1.75M KCl, D. S synthetic defined medium-ura-met + 1.5M NaCl

No significant difference was found between cells expressing either of the phosphomutants (S90A and S90D) and cells expressing the wild type version of the protein.

4.3. Binding of Vps45p to *in vitro* SNARE complexes containing Tlg2p mutants

4.3.1. Preparation of SNARE complexes containing Tlg2p (WT, S90A, S90D), Tlg1p, Vti1p and Snc2p proteins

In this study we tried to assemble a functional complex of Tlg1p, Vti1p, Snc2p and Tlg2p (WT) and also complexes made from the same SNARE proteins but instead of Tlg2p (WT), we made complexes with Tlg2p (S90A) and Tlg2p (S90D). We co-expressed plasmid DNA encoding Tlg1cyto and His tagged-Snc2cyto with plasmid DNA encoding Vti1cyto and PrA tagged Tlg2cyto-WT or S90A or S90D in bacteria cells BL21 and then we induced the protein expression by adding IPTG. All of the SNAREs in these complexes lacked their transmembrane domains. The complex of proteins was purified by using affinity of PrA tag on Tlg2cyto to IgG Sepharose. The assembled complexes were then cleaved from the IgG sepharose using a thrombin-cleavage site between PrA tag and Tlg2cyto. The cleaved complex was then purified using the His tag on His-Snc2cyto to Ni-NTA agarose. As a reference sample we expressed in the same bacteria strain just PrA tagged empty vectors (Fig. 14).

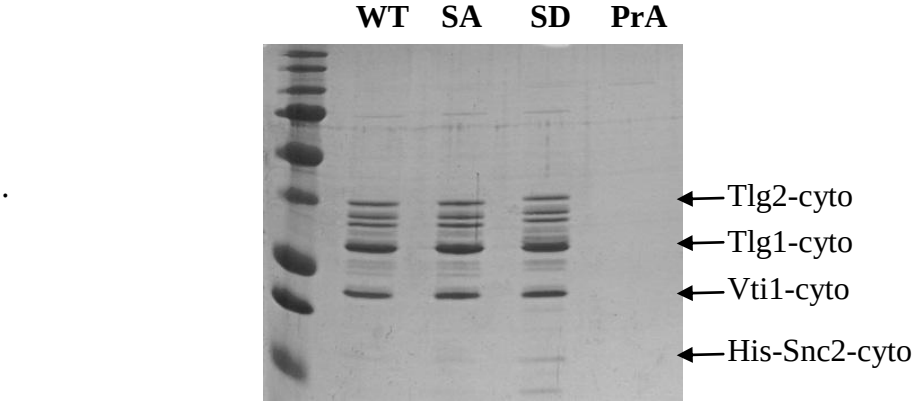

Figure 14: SNARE complex expression
Co-expression of plasmid DNA encoding Tlg1cyto and His-Snc2cyto with plasmid DNA encoding Vti1cyto and Tlg2cyto WT or S90A (SA) or S90D (SD) in bacteria cells BL21, and then induction of the protein expression by adding IPTG. The complex of proteins was purified by using affinity of PrA tag to IgG Sepharose and His tag to Ni-NTA Agarose (both of the tags are part of the complex). PrA tagged empty vector expressed in the same bacteria strain and purified by using same way was used as a reference sample. Samples of protein complexes were run on 15% gel using SDS-PAGE and visualized by Coomassie.

Fig. 17 shows that we made protein complexes containing Tlg2-cyto WT, S90A or S90D with Tlg1-cyto, Vti1-cyto and Snc2-cyto.

In order to check whether the complexes were assembled correctly and contained the 4 SNARE proteins Tlg2p, Tlg1p, Vti1p and Snc2p, we run the samples containing complexes on 10% SDS-Page gel and the presence of our 4 different SNARE proteins was assessed using Western immunoblot analysis with an antibodies specific to these SNARE proteins (Fig. 15).

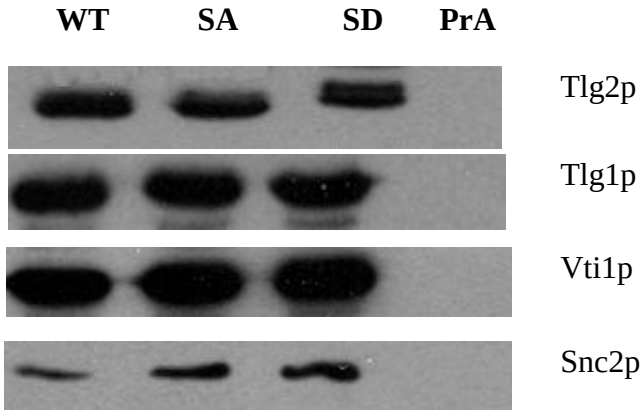

Figure 15: Control of in vitro SNARE complexes made by Tlg2p (WT, S90A, S90D), Tlg1p, Vti1p and Snc2p
Co-expression of plasmid DNA encoding Tlg1cyto and His-Snc2cyto with plasmid DNA encoding Vti1cyto and Tlg2cyto WT or S90A (SA) or S90D (SD) in bacteria cells BL21, and then induction of the protein expression by adding IPTG. The complex of proteins was purified by using affinity of PrA tag to IgG Sepharose and His tag to Ni-NTA Agarose (both of the tags are part of the complex). PrA tagged empty vector expressed in the same bacteria strain and purified by using same way was used as a reference sample. Samples were run on 10% SDS-PAGE gel and the presence of our 4 different SNARE proteins was assessed using immunoblot analysis whit an antibodies specific to these SNARE proteins: anti-Tlg2p, anti-Tlg1p, anti-Vti1p and anti-Snc2p.
SA – mutant S90A, SD – mutant S90D

The SNARE complex containing Tlg2p wild type, S90A or S90D mutants along with Tlg1p, Vti1p and Snc2p was successfully made.

4.3.2. Binding of Vps45p to *in vitro* SNARE complexes containing Tlg2p (WT, S90A or S90D) Tlg1p, Vti1p and Snc2p

The aim of this work was to try to bind protein Vps45p to SNARE complexes containing Tlg2p S90A and S90D mutants. As was mentioned before, the mechanism involved in the binding of Vps45p to Tlg2p has a different mechanism compared to binding to Tlg2p in SNARE complexes. In binding assays to Tlg2p alone (not in complex with other SNAREs), there were no significant differences between both mutants and WT so we wanted to try binding to *in vitro* complexes. Therefore we made *in vitro* 3 sets of complexes as follows. First was made from Tlg2p-cyto (WT), Tlg1p-cyto, Vti1p-cyto and Snc2p-cyto, second containing Tlg2p-cyto (S90A), Tlg1p-cyto, Vti1p-cyto and Snc2p-cyto and third containing Tlg2p-cyto (S90D), Tlg1p-cyto, Vti1p-cyto and Snc2p-cyto.

We carried out a pull-down assay using the purified complexes and Vps45p from yeast cell lysates and the resulting samples were run on 10% SDS-PAGE gel and visualised using anti-Vps45p or anti-HA antibody (as the Vps45p in our assay had an HA tag) (Fig. 16).

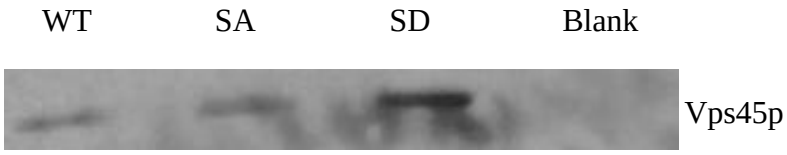

Figure 16: Pull-down assay – interaction between our SNARE complexes and Vps45p. Interaction between SNARE complexes containing Tlg2p WT or S90A or S90D, Tlg1p, Vti1p, Snc2p and between Vps45p (HA tagged). We used pull-down assay as is written above in section 3.2.12. Samples were separated by using SDS-PAGE Electrophoresis and visualised by using immunoblot with anti-HA antibody. Blank Ni-NTA beads were used as a binding control.
SA – mutant S90A, SD – mutant S90D

The result of this assay indicates that SNARE complex containing phosphomimetic mutant of Tlg2p (S90D) along with, Tlg1p, Vti1p and Snc2p binds Vps45p better than similar complexes containing Tlg2p wild type or S90A mutant.

4.4. Binding of Vps45p to *in vitro* SNARE hybrid complexes containing mammalian Stx16 and yeast Tlg1p, Vti1p and Snc2p

4.4.1. Preparation of hybrid SNARE complex containing mammalian syntaxin 16 and Yeast Tlg1p, Vti1p, Snc2p

It is known that syntaxin 16 is functional homologue of Tlg2p. To address whether syntaxin 16 is homologous to Tlg2p in complexes, it was useful to express human syntaxin 16 in *S. cerevisiae* and investigate whether it could perform the same functions as Tlg2p *in vivo*. It was demonstrated that expression of syntaxin 16 in yeast cells lacking endogenous Tlg2p complements many of the trafficking phenotypes displayed by yeast cells lacking Tlg2p [90].

The aim of this part of study was try to make an *in vitro* hybrid-SNARE complex containing mammalian SNARE protein syntaxin 16 and yeast SNARE proteins Tlg2p, Vti1p and Snc2p.

For cloning of plasmid containing syntaxin16-cyto-PrA + Vti1-cyto we used plasmid pET-Duet - WT Tlg2cyto-PrA + Vti1cyto. We cut Tlg2-cyto out of the plasmid using restriction endonucleases NdeI and XhoI. The same endonucleases we used for cutting of plasmid pVT102u - HA.STX16 (WT). Then we purified fragments from agarose gel and ligate syntaxin 16-cyto fragment into plasmid containing Vti1-cyto.

We co-expressed plasmid DNA encoding Tlg1-cyto and His-Snc2-cyto with plasmid DNA encoding Vti1-cyto and PrA-syntaxin16-cyto (WT) in *E. coli* BL21 cells and then induced protein expression by adding IPTG. The complex of proteins was purified by using affinity of PrA tag to IgG Sepharose and His tag to Ni-NTA Agarose (both of the tags were part of the complex). As a reference sample we expressed in the same bacteria strain just PrA tagged empty vectors (Fig. 17, page 61).

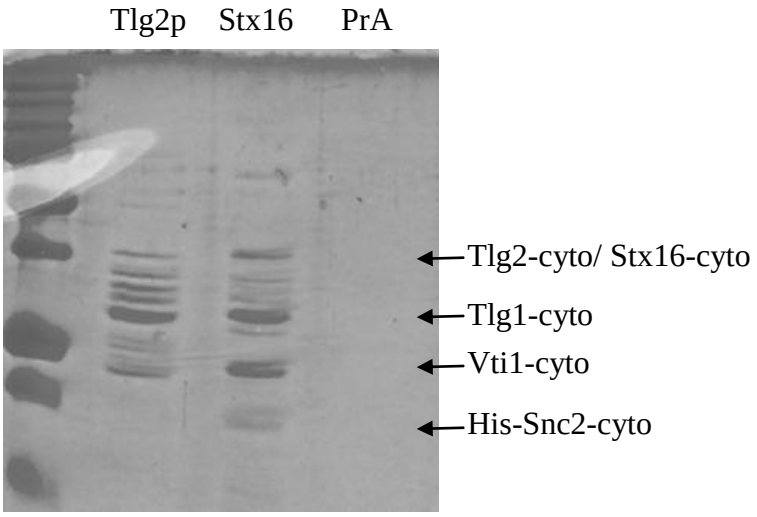

Figure 17: SNARE complex expression
Co-expression of plasmid DNA encoding Tlg1cyto and His-Snc2cyto with plasmid DNA encoding Vti1cyto and Tlg2cyto WT or syntaxin 16 WT (Stx16) in bacteria cells BL21, and then induction of the protein expression by adding IPTG. The complex of proteins was purified by using affinity of PrA tag to IgG Sepharose and His tag to Ni-NTA Agarose (both of the tags are part of the complex). As a reference sample was used just PrA tagged empty vectors expressed in the same bacteria strain and purified by using same way. Samples of protein complexes were run on 15% gel using SDS-PAGE and visualized by Coomassie.

As can be seen (Fig. 17), it was possible to make *in vitro* SNARE complexes containing mammalian SNARE protein syntaxin 16 with other 3 yeast SNARE proteins Tlg1p, Vti1p and Snc2p.

4.4.2. Binding of Vps45p to the hybrid SNARE complex containing synatxin 16, Tlg1p, Vti1p and Snc2p

The aim of this part of study was to try to bind Vps45p to the assembled SNARE complex containing syntaxin 16 wild type and yeast SNAREs Tlg1p, Vti1p and Snc2p. Therefore we made *in vitro* complex from syntaxin 16 WT, Tlg1p, Vti1p and Snc2p. We carried out a pull-down assay using the hybrid SNARE complex with syntaxin 16-cyto and Vps45p from *S. cerevisiae* and the resulting samples we run on 10% SDS-PAGE gel and visualised using antiVps45p or antiHA antibody (as the Vps45p in our assay had an HA tag) (Fig. 18, page 62).

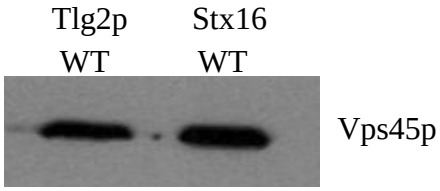

Figure 18: Pull-down assay – interaction between our SNARE complexes and Vps45p. Interaction between SNARE complexes containing Tlg2p WT or syntaxin 16 along with Tlg1p, Vti1p, Snc2p and between Vps45p (HA tagged). We used pull-down assay as is written above in section 3.2.12. Samples were separated by using SDS-PAGE electrophoresis and visualised by using immunoblot with anti-HA antibody. As a reference sample was used just PrA tagged empty vector.
Stx16 – syntaxin 16

In a pull-down assay was shown that hybrid SNARE complex containing mammalian SNARE protein syntaxin 16 and yeast SNARE proteins Tlg1p, Vti1p and Snc2p is able to bind yeast SM protein Vps45p at a similar level as normal yeast SNARE complex made from Tlg2p, Tlg1p, Vti1p and Snc2p.

5. Discussion

The first aim of this study was discover whether it was possible to express Tlg2p mutants S90A (serine 90 is mutated to alanine, mutant cannot be phosphorylated) and S90D (serine 90 is mutated to aspartic acid, mutant is phosphomimetic – acid carboxyl group mimics phosphate group) in yeast cells lacking endogenous Tlg2p. It was already known that cells lacking endogenous Tlg2p are viable but these cells display a variety of different phenotypes representative of defects in endocytosis [63], [64], [65]. The follow up set of experiments was to check if expression of the Tlg2p mutants in cells lacking endogenous Tlg2p can restore the trafficking defects and normal function of endocytic pathway in yeast. We expressed them in yeast cells lacking endogenous Tlg2p. We tried to express these Tlg2p mutants in two different *S. cerevisiae* strains. SF838-9D was the first strain and RPY10 was the second one. The difference between these congenic strains is that RPY10 contains the vacuolar protease *PEP4* and SF838-9D is harboring the *pep4-3* mutation. Mutation of the *PEP4* gene results in the loss of vacuolar activity as *PEP4* encodes an aspartyl protease that is required to activate vacuolar hydrolases [95]. The expression of Tlg2p serine mutants in both strains was successful. However in strain SF838-9D the expression of mutant S90D was always higher. Since all three versions are expressed from the same promoter, it appears that the higher cellular levels of the Tlg2p-S90D mutant reflect decreased degradation of the protein.

In order to look for functional differences due to Tlg2p phosphorylation status, it was necessary to express all of the mutants at an equivalent level. In previous studies it had been shown that SNARE interactions can be promiscuous as a result of the many coiled-coil sequences they contain that make them very “sticky”, so if over-expressed they can also interact with other non-cognate SNARE proteins [23], [24], [25]. In the yeast strain RPY10 the wild type and both mutants had a similar level of expression. This leads us to the conclusion that the higher cellular levels of S90D are due to loss of function of vacuolar proteases in *pep4-3* strain SF838-9D. From work of Marion Struthers [96] we know that Tlg2p in wild type cells is degraded by vacuolar proteases (Fig. 19, page 64).

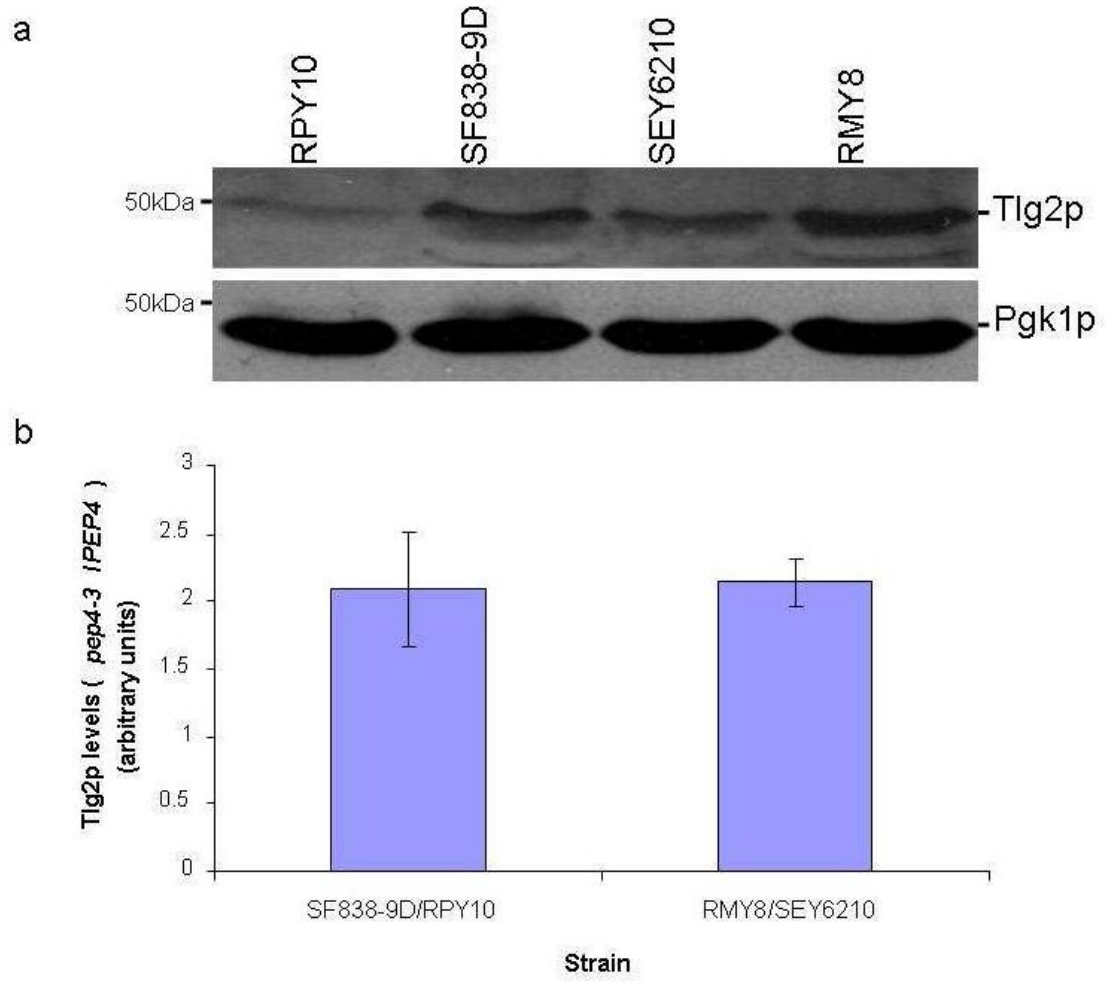

Figure 19 – Cellular levels of Tlg2p are elevated in cells lacking vacuolar activity. Immunoblot analysis was used to compare the steady-state levels of Tlg2p in wild type cells containing active vacuolar proteases (RPY10 and SEY6210) and those lacking vacuolar proteases (SF838-9D and RMY8). (a) Proteins contained within whole cell lysates, prepared from 1 OD600 equivalents, were separated using SDS-PAGE prior to immunoblot analysis with an anti-serum specific to Tlg2p. The same filter was probed for Pgk1p to control for equal loading. b) The levels of Tlg2p detected by immunoblot analysis were quantified using the computer software, Image Processing and Analysis in Java (Image J) and normalized to the loading control Pgk1p. The ratio of Tlg2p in cells lacking active vacuolar proteases (*pep4-3*) and those containing vacuolar proteases (*PEP4*) was then calculated (*pep4-3/PEP4*). Error bars are '+' and '-' standard deviations of the mean, n=3 (taken from [96]).

Figure 19 shows that steady-state levels of Tlg2p in wild type cells lacking active vacuolar proteases are increased approximately 2 fold when compared to their isogenic strains containing active vacuolar protease (2.03 ± 0.43 fold greater in SF838-9D than RPY10 and 2.14 ± 0.18 fold greater in RMY8 than SEY6210) [96].

Bryant & James (2001) [74] and Struthers (2010) [96] also found that proteasomal degradation of Tlg2p is independent of Pep4p-dependent vacuolar degradation. In the absence of Pep4p, the role of the proteasome in the degradation of Tlg2p is revealed.

The vacuolar protease Pep4p is the most important difference between both strains we used in this assay. From my results, it appears that phosphorylation of Tlg2p at serine 90 or the mutation Tlg2p-S90D may play some role in protecting Tlg2p from non-Pep4-dependent degradation, possibly by the proteasome.

On the other hand in strain lacking endogenous Vps45p, the t-SNARE Tlg2p is degraded rapidly by the proteasome [74]. In Fig. 10 (page 53) it can be seen that there are significantly lower cellular levels of Tlg2p in the strain lacking Vps45p compared to the strain lacking Tlg2p. This is due to proteosomal degradation, as both strains lack the vacuolar protease *PEP4*. However, there is no significant difference in cellular levels of Tlg2p between the Tlg2p-phosphomutants and the wild type Tlg2p protein in Δ*vps45* cells. This suggests that Vps45p protects Tlg2p from proteosomal degradation, and that in the absence of *PEP4*, phosphorylation of Ser90 on Tlg2p or the S90D phosphomimetic mutation may stabilise the interaction between Tlg2p and Vps45p, and therefore stabilise Tlg2p levels.

To find out whether the Tlg2p Ser90-mutants are able to rescue Δ*tlg2* cell phenotypes caused by loss of endogenous Tlg2p, we did two different assays. Analysis of carboxypeptidase Y trafficking in cells containing the mutants Tlg2p-S90A and Tlg2p-S90D was the first one. This is based on the fact that yeast cells lacking endogenous Tlg2p missort 15-20 % of carboxypeptidase Y on the way to the vacuole [63] and the second assay was to check whether the mutants can rescue Δ*tlg2* cells from salt/osmotic stress sensitivity as is known that yeast cells lacking endogenous Tlg2p suffer from salt/osmotic stress [63].

From our results it can be seen that in yeast strain SF838-9D, approximately the same amount of carboxypeptidase Y is secreted while yeast contain Tlg2p (WT) and Tlg2p (S90A), but the Tlg2p (S90D) mutant secretes lower amounts of carboxypeptidase Y. However in the yeast strain RPY10 there is no significant difference between yeast cells containing Tlg2p (WT), and mutants S90A and S90D. As was written previously, Tlg2p has a different stability in these two strains due to degradation by vacuolar proteases and the proteasome. In yeast strain SF838-9D, S90D mutant of Tlg2p is more stable (less degraded = stronger expression is seen) than in yeast strain RPY10. So it looks like the different stability (different cellular levels of Tlg2p) caused different levels of missorted/secreted carboxypeptidase Y (stronger expression = better complementation of missorting/secretion of carboxypeptidase Y). The conclusion is that phosphorylation of Tlg2p at serine 90 does not play a significant role in

sorting of carboxypeptidase Y. Both phosphomutants were able to complement the loss of endogenous *TLG2* in this assay.

As described previously, cells lacking endogenous Tlg2p show a failure to grow on high salt medium [63]. From our results you can see that we confirmed this, and found that expression of Tlg2p(WT), Tlg2p (S90A) or Tlg2p (S90D), were all able to restore growth on high salt plates. There was no significant difference between the wild type Tlg2p and the S90A or S90D mutants.

Next part of this work was to test if phosphorylation at putative phosphorylation site serine 90 has some influence on binding Vps45p to whole SNARE complex containing Tlg2p, Tlg1p, Vti1p and Snc2p. It was demonstrated that Vps45p binds to monomeric state of Tlg2p before the formation of the trans-SNARE complex, and then it dissociates and is released into the cytosol. The conversion of trans-SNARE complex to the cis-configuration defines membrane fusion and it is in this time when Vps45p reenters the cycle by binding to the cis-complex [87]. From our results can be seen that Vps45p binds stronger to the SNARE complex containing S90D mutant (phosphomimetic) of Tlg2p than to the SNARE complexes containing S90A mutant of Tlg2p or wild type.

These data backs up the previous results showing better stability of Tlg2p-S90D in SF838-9D. It looks like Vps45p protects Tlg2p from proteosomal degradation (which was also reported by [74] and [96]). In SF838-9D when there is no pep4 and vacuolar degradation is removed, the effect of the proteasome can be seen more clearly. The higher levels of Tlg2p-S90D may suggest that it binds to Vps45p tighter and therefore is protected from the proteasome more than the Tlg2p-WT and Tlg2p-S90A mutant.

Next goal of this thesis was to try to construct hybrid SNARE complex containing mammalian syntaxin 16 and yeast SNAREs Tlg1p, Vti1p and Snc2p. It is known that syntaxin 16 is structural and functional homologue of Tlg2p which works usually in this SNARE complex as a syntaxin (Qa). Therefore, we tried to construct this hybrid complex in yeast. As you can see from our results, we were successful. The formation of the syntaxin 16 hybrid complex was done for the first time ever and it is very useful for next investigation of how is the mammalian SNARE protein syntaxin 16 working. We also tested if this complex can binds Vps45p. On Fig. 21 it can be seen that hybrid complex containing syntaxin 16 can bind Vps45p at a same level as usual yeast SNARE complex with Tlg2p.

Three putative phosphorylation sites were identified on syntaxin 16 based on the phosphorylation site found on Tlg2p. Syntaxin 16 and Tlg2p have homologous sequence. The phosphorylation site on Tlg2p is serine 90. Syntaxin 16's three putative phosphorylation sites

around residue 90 are Threonine 90, Serine 94 and Serine 95 (Fig. 20). The following research will be focused on creation of a set of protein expression constructs containing putative phosphorylation site mutant forms of Syntaxin 16 based on homology with Tlg2p and checking whether Vps45p binding to SNARE complexes is enhanced with phosphomimetic mutations of syntaxin 16.

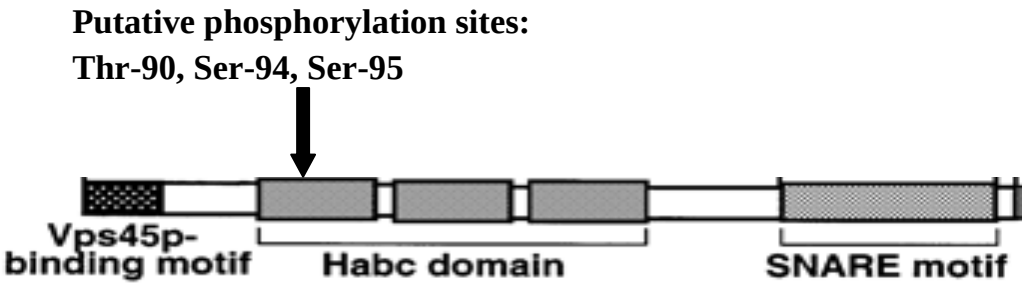

Figure 20: Diagram of structure of gene for Stx16 with 3 putative phosphorylation sites shown on.

6. Summary

In this work, we were trying to find some functional phenotypes of Tlg2p based on its phosphorylation site at serine 90. We used two mutants, S90A (cannot be phosphorylated) mutant and phosphomimetic S90D mutant. We observed that both of these mutants can be expressed in yeast strains SF838-9D and RPY10, however in strain SF838-9D which is lacking vacuolar protease Pep4p, Tlg2p-S90D is expressed at higher level. We found out that neither S90A mutant nor S90D mutant show any significant difference from wild type in carboxypeptidase Y missorting phenotype or salt/osmotic stress sensitivity phenotype.

In the next part, we showed that binding of Vps45p to SNARE complex containing Tlg2p, Tlg1p, Vti1p and Snc2p is influenced by phosphorylation of Serine 90 on Tlg2p. SNARE complex with phosphomimetic mutant of Tlg2p (S90D) was able to bind Vps45p better that complexes containing mutant S90A or wild type of Tlg2p.

Last part of this work showed that it is possible to make hybrid SNARE complex containing mammalian protein syntaxin 16 and three yeast proteins Tlg1p, Vti1p and Snc2p and that this complex is able to bind Vps45p at a same level as a normal yeast SNARE complex made from Tlg2p, Tlg1p, Vti1p and Snc2p.

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