CHARACTERIZATION OF VPS6P
AND THE DETERMINATION OF ITS PROTEIN PARTNERS

by

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Vps6p is a lock protein located on the prevacuolar compartment in *Saccharomyces cerevisiae* that regulates the docking and fusion event of Golgi-derived vesicles with the compartment. In order to further characterize the function of Vps6p, I isolated temperature-sensitive *vps6* mutants. All rapid-onset temperature-sensitive alleles coded for proteins that lacked transmembrane domains. These mutants continued to properly sort carboxypeptidase Y to the vacuole at the permissive temperature and even at the nonpermissive temperature when overexpressed. It is possible that an accessory protein provides a binding site for Vps6p and aids in the regulation in the docking and fusion event. A suppression screen was designed to identify this putative accessory protein.

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Introduction

Before I describe my research, I would first like to introduce the eukaryotic microorganism I work with: the yeast, *Saccharomyces cerevisiae.* Eukaryotic cells are cells that have compartments, and particularly a nucleus. Prokaryotes, by contrast, lack this division and contain all biological contents within one casing. For the most part, eukaryotes are multicellular organisms, such as plants and animals. Yeast, as a single celled organism, is one of the simplest eukaryotes and can be genetically manipulated with ease. This is why yeast is such a popular research model organism.

Eukaryotic cells are divided into individually regulated compartments called organelles. Organelles are distinct, each having a specific lipid bilayer composition, membrane proteins, and inner content. A lipid bilayer is a bimolecular sheet of phospholipid molecules, where one end of each phospholipid is in contact with the aqueous environment while the other ends are backed up to one another. A membranous border defines the discrete area of an organelle. The flux of proteins in and out of an organelle is controlled by the membrane and associated proteins that act as "customs" between two very diverse, yet neighboring environments. To maintain several biochemically and enzymatically autonomous environments within a cell, the mechanism of delivering compartment-specific proteins must be precise.

Proteins transported through the secretory pathway within yeast cells pass through two organelles, the endoplasmic reticulum (ER) and the Golgi apparatus (Golgi) as seen in Figure 1. Soluble proteins, ER-resident proteins, and proteins destined for the vacuole are first synthesized on the ER (Voet and Voet, 1995). Upon translocation into the ER, the proteins enter the secretory pathway. The proteins leave the ER and enter the next organelle, the Golgi. The Golgi has three individual compartments, called the *cis*, *medial*, and *late*

Golgi. As a protein passes through each of the Golgi compartments, the protein is chemically modified. Due to this modification, a protein that has been transported through the Golgi can be distinguished from the original protein synthesized in the ER.

A protein can follow three paths after leaving the final Golgi compartment as depicted in Figure 1. Secreted proteins are packaged into vesicles (small, membrane-enclosed carriers that bud off the original compartment) and are sent to the plasma membrane (the edge of the cell) where they are extruded from the cell. By contrast, vacuolar proteins diverge from secreted proteins at the late Golgi. Vacuolar proteins, which depart from the late Golgi in vesicles, take one of two pathways. One pathway, called the Carboxypeptidase Y (CPY) pathway, transports proteins through an intermediate compartment called the prevacuolar compartment (PVC) before reaching the vacuole (Bryant and Stevens, 1998). By contrast, the other vacuolar pathway bypasses the PVC and transports proteins directly to the vacuole.

To attack the question of how protein transport is regulated within the cell, I have focused my attention on one particular system, the CPY pathway. As I previously mentioned, the transport of vacuolar proteins in the CPY pathway is not direct, having an intermediate step between transport from the late Golgi to the vacuole. My research concentrates on the regulation of transport through the intermediate step, or PVC. The PVC is known to receive traffic directly from the late Golgi and via endocytosis. During endocytosis, the plasma membrane folds inward into the cell creating vesicles that transport extracellular proteins inside.

One protein that follows the pathway through the PVC from the ER to the vacuole is the vacuolar protein carboxypeptidase Y (CPY). At each compartment in the pathway, CPY is chemically modified by enzymes specific to

the compartment. CPY has three forms: ER modified (p1), Golgi modified (pro), and mature (m). Due to this modification, the progression of CPY through various organelles can be followed. A receptor recognizes CPY at the Golgi and directs its transport to the PVC (Cooper and Stevens, 1996). The delivery of CPY to the vacuole is disrupted when the function of a vacuolar protein sorting (*VPS*) gene is compromised, which results in the secretion of proCPY. Although CPY is not sorted properly, the cell is still viable, signifying that the *VPS* genes are not essential.

Several classes of *VPS* genes have been established based on various morphological irregularities that arise when the gene loses function as a result of mutation. Morphological irregularities include enlarged vacuoles, exaggerated prevacuolar compartments, or an accumulation of vesicles (Raymond et al., 1992). As a result of these morphological characteristics, the step in the pathway at which the product of a particular *VPS* gene functions can be identified.

One of these morphological classes, known as Class D, is identified by the accumulation of Golgi-derived transport vesicles in a loss-of-function mutant strain. The protein products of Class D genes allow for the docking and fusion of the Golgi-derived vesicles with the PVC (Conibear and Stevens, 1995; Horazdovsky et al., 1995). In Class D mutants, the transport of proteins is blocked after vacuolar proteins leave the Golgi in vesicles and before entry into the PVC, causing a characteristic accumulation of Golgi-derived vesicles. Due to the accumulation of trapped vesicles carrying CPY and its receptor, the level of the receptor returning to the Golgi drops such that CPY passing through the Golgi is not packaged into vesicles destined for the PVC and is instead loaded into secretory vesicles *en route* to the plasma membrane. Therefore, interfering with CPY receptor recycling leads to CPY secretion. The protein products of

three Class D genes have been identified as homologues of proteins known to be involved in the secretion of neurotransmitters in animals (Bennet and Scheller, 1993). One of these proteins is Vps6p.

Neurons, or nerve cells, form a chain-like assembly of cells that connect the nervous system to other organs. This assembly of neurons conducts electrical impulses or signals throughout the body. Between two nerve cells lies a synapse, or nerve cell junction, through which electrical impulses pass (Voet and Voet, 1995). In order to transmit electrical impulses across the junction, synaptic vesicles rapidly fuse with the plasma membrane of the nerve cell and release into the synapse large amounts of neurotransmitters that are detected by the next neuron. The electrical signals transferred through your body allow you to react quickly to stimuli. Because yeast have proteins homologous to those involved in neurotransmitter release, what is learned from Class D genes may increase the understanding of the mechanism of neuronal secretion.

Proteins located on transport vesicles (such as synaptic vesicles) and target membranes (such as the plasma membrane in the case of the synaptic vesicles) are required for membrane fusion (Rothman, 1994). In order to visualize the protein interaction, imagine the vesicle and the compartment as two rooms separated by a closed door. The door can be opened only if the key fits the lock, and likewise, the membranes can fuse only if the correct protein on the vesicle fits the counter-part on the compartment. Once this recognition occurs, the door is opened, and the areas of the two rooms are combined, just as the contents of a compartment and a vesicle are combined by membrane fusion. Once the door is opened, the person passes from one room into the next freely. Similarly, a protein moves between the vesicle and compartment. The necessity for a specific key to open a door is analogous to how the recognition between vesicle and compartment proteins is essential for protein

trafficking.

My research focuses on the function of the gene *VPS6*, which encodes Vps6p, a lock protein located on the membrane of the PVC (Becherer et al., 1996). Figure 2 depicts Vps6p, as a lock protein, working in conjunction with a key protein on a Golgi-derived vesicle and an accessory protein. I chose this particular lock, Vps6p, because it acts at the PVC where the pathways of protein entry from the cell surface and the CPY pathway converge as seen in Figure 1. This lock has the potential to be a component in one pathway and not the other, or alternatively could function in both pathways, being a lock with more than one key.

Materials and Methods

Mutagenesis

Within yeast, there are two types of DNA, genomic and plasmid DNA. Genomic DNA originates in the yeast cell, is organized into chromosomes, and controls the manufacturing and function of proteins, thereby controlling the function of the cell. Plasmid DNA, on the other hand, is variable and can be removed from one cell and transferred (transformed) into another. Although a plasmid is a ring of DNA extraneous from the genomic DNA, the genetic products of both can function together within the same cell.

I mutagenized a plasmid containing the *VPS6* gene. Mutagenesis alters the original DNA sequence of a gene to create errors. To mutagenize the plasmid, hydroxylamine was employed. Hydroxylamine is a chemical mutagen that deaminates cytosine (one type of nucleotide that constitutes the DNA sequence) and converts it to a compound that base pairs with adenine (another type of nucleotide) (Voet and Voet, 1995).

The plasmid containing *VPS6* was prepared using a kit (as described in

the manufacturer's instructions, Qiagen) and was mutagenized using 0.36 M hydroxylamine in 0.05 M potassium phosphate pH6. After incubating the sample at 70°C for 0, 30, 45, or 60 minutes, 10 µl of 3 M sodium acetate pH 4.8 was added. The sample was briefly vortexed and 250 µl of 100% ethanol was added. The sample was inverted to mix and spun in a table top centrifuge for 15 minutes at 4°C. The pellet washed with 70% ethanol and resuspended in 100 µl water. The precipitate procedure was repeated two times after which the pellet was resuspended in 20 µl water. Using 4 µl of the mutagenized plasmid, *Escherichia coli* (*E. coli*) were transformed by electroporation.

Temperature Sensitive Experiments

After the mutagenized plasmid was purified, it was transformed (inserted) into a loss-of-function *VPS6* yeast strain (*vps6Δ*). Yeast *vps6Δ* cells lack a copy of *VPS6* . To determine which alleles (versions of a gene that have a specific mutation) were temperature sensitive for function (*vps6ts*), the level at which each mutant secreted CPY was determined at two temperatures, 25°C and 37°C. A temperature sensitive mutant is one that doesn't secrete CPY (correlated with normal function) at 25°C, yet secretes this protein heavily (correlated with compromised function) at 37°C.

The mutagenized plasmid library (a collection of mutagenized plasmids) was polyethylene glycol precipitated and transformed into the *vps6Δ* yeast strain. The cells transformed with the mutagenized library were plated on selective media and grown at 25°C. The density of the cells was approximately 300 colonies per plate. The colonies were replica plated onto selective media,

overlayed with a nitrocellulose filter and incubated at 37°C. As CPY is secreted from the cell, it sticks to the nitrocellulose filter. The filter was probed for the presence of CPY after 14 hours of incubation using an antibody for CPY. The colonies that secreted CPY heavily at 37°C were selected and patched onto fresh plates with controls: *VPS6* wildtype and *vps6Δ* mutant. The selected mutants were grown at 25°C and were then replica plated onto selective media twice. The identical plates were overlayed with a nitrocellulose filter, one was incubated at 25°C and the other was incubated at 37°C. The filters were probed for the presence of CPY. Each potential mutant was struck for single colonies, and using another CPY overlay at the two temperatures were identified as clonal. The plasmid was extracted and transformed in XLII *E.coli*, using electroporation. The colonies were grown on selective media at 37°C. The plasmid was purified, transformed into the *vps6Δ* mutant strain, and grown on selective media at 25°C. A colony for each transformed mutant was patched onto selective media with the previously mentioned controls and the original colony and probed for CPY secretion at 25°C and 37°C.

Pulse/chase Immunoprecipitations

Pulse/chase immunoprecipitation is a process of tracking the fractions of CPY that lie internally and externally to the cells at various time points. First a "pulse" of radioactive label is added to the medium containing the cells. After allowing time for the cells to internalize the label and incorporate it into new proteins, the "pulse" is "chased" with cold methionine and cysteine and CPY is immunoprecipitated.

Newly synthesized CPY in *vps6ts* cells was followed using immunoprecipitation of CPY. Yeast cultures were grown overnight at 25°C in selective media to an optical density (OD) of 600 = 1.0. For each time point to be immunoprecipitated, an OD600 = 0.5 was taken, pelleted, and resuspended in fresh media. To stabilize the temperature, the cells were first incubated at 25°C. Half the cells for each time point were preincubated at 37°C for 15 minutes. The cells were pulsed for 10 minutes by the addition of 100 μCi [35S]-Express label per OD600 = 0.5 of cells. The label was then chased with cold methionine and cysteine at time points of 0 and 60 minutes for cells at 25°C and 0 and 40 minutes for cells at 37°C.

Western blot

In a western blot, proteins are extracted from a yeast culture and run through a gel by electrophoresis. Electrophoresis is a process that uses an electric potential to separate the proteins based on size. Proteins have a net negative charge as a result of the negatively charged detergent in the buffer and therefore travel from the negatively charged cathode to the positively charged anode. The smaller a protein, the faster it travels through the gel. All of the proteins can then be transferred from the gel onto a filter and the desired protein can then be detected using an antibody specific to that protein. It is possible to determine the size of a protein by comparing the distance it traveled with the distance traveled by a marker protein of known size. In this case, the filter was probed with an antibody specific to Vps6p.

The potential temperature-sensitive mutants and controls (wildtype and *vps6Δ*

mutant strains) were grown on selective media at 25°C overnight. The following day, rich medium was added to the overnight cultures and grown for approximately two cycles (4 hours). Once the cells reached an OD600=1.0, the cells were pelleted. The pellet was resuspended in 1 ml water and transferred to an Eppendorf tube. The cells were repelleted, by spinning for 2 minutes in the table top centrifuge at 13200 rpm. After decanting the supernatant, 180 µl Thorner sample buffer and 20 µl ß-mercapthoethanol were added. In addition, a half cap full of glass beads was added to lyse the cells while vortexing for 5 minutes. The cellular debris was pelleted for 5 minutes at 13,200 rpm. The supernatant, of each of the temperature sensitive mutants and a control, was loaded into the lanes of a polyacrylamide gel. In addition, a high molecular weight marker was loaded into the first lane of the gel. The control was a protein sample from *VPS6* cells. The gel was run at 100 volts.

After the dye front reached the end of the gel, the electric current was turned off. A piece of wet blotting paper was placed on the foam holder of the transfer apparatus. The gel was placed on top of the blotting paper and covered by a piece of wet nitrocellulose. The nitrocellulose and gel were sandwiched by another piece of wet blotting paper. Once the apparatus was set up, a current transferred the proteins from the gel onto the nitrocellulose.

The proteins were transferred to the nitrocellulose and it was stained with Ponceau S. The filter was shaken for 20 minutes in 5% milk in TTBS (Tris, Tween, buffered saline) and incubated with a Vps6p antibody for 2 hours. The filter was then washed and incubated with a rabbit antibody horseradish peroxidase (HRP) for 2 hours. The filter was washed a second time. After swirling the filter in 1X phosphate buffered saline (PBS), the filter was developed using chemiluminescent reagents and exposed to film.

Suppression Screen

A genomic library was transformed into the *vps6ts* yeast strain. A genomic library is a collection of overexpression plasmids that contain pieces of DNA that together represent the entire yeast genome. Overexpression is having many copies of the gene of interest inside each cell, which then produce the protein product at higher levels. Within the *vps6ts* strain, the only copy of *VPS6* produced the truncated form of the protein. To determine which genes from the library suppressed the temperature-sensitive function of *vps6ts*, the level of CPY secreted by each transformant at 37°C was observed. In this case, a suppressor was defined as a gene contained on an overexpression plasmid that overcomes typical secretion of CPY by the temperature-sensitive mutant at 37°C.

To identify multicopy suppressors that allow for wildtype function of the temperature-sensitive mutant at 37°C, I transformed 9000 *vps6ts* cells with a YEp24 2μ library (Carlson and Botstein, 1982). The transformants were then plated on selective media and grown at 25°C. The density of the cells was approximately 300 colonies per plate. The colonies were replica plated on selective media, overlayed with a nitrocellulose filter and incubated at 37°C. The filter was probed for the presence of CPY after 14 hours of incubation. The colonies with low levels of CPY secretion at 37°C were selected and patched with controls: overexpressed *vps6ts*, overexpressed *VPS6*, overexpressed *VAM3*, and *vps6ts* mutant. The selected transformants were grown at 25°C and were then replica plated onto selective media. The plates were overlayed

with a nitrocellulose filter and incubated at 37°C. The filters were probed for the presence of CPY. Each potential suppressor was struck for single colonies, and using another CPY overlay at 37°C were identified as clonal. The plasmid was extracted and transformed in XLII *E.coli*, using electroporation. The colonies were grown on selective media at 37°C. The plasmid was purified, transformed into the *vps6ts* strain, and grown on selective media at 25°C. A colony for each new transformant was patched onto selective media with the previously mentioned controls and the original colony and probed for suppression of CPY secretion at 37°C.

In summary, a plasmid containing *VPS6* was first mutagenized and the resulting alleles were screened to find a temperature sensitive mutant by observing the levels of CPY secretion at two temperatures. A pulse/chase immunoprecipitation was performed to find the fraction of CPY retained within the cell and the fraction of CPY secreted from the cell. In addition, a western blot was performed to determine the size of the protein encoded by the temperature sensitive alleles containing a stop codon upstream of the transmembrane domain. And finally a screen was begun in hopes of uncovering a gene that suppresses the distorted function of the temperature sensitive allele at 37°C.

Results

Isolation of temperature sensitive mutants

A plasmid containing the *VPS6* gene was mutagenized using hydroxylamine. Once the plasmid was mutagenized, it was transformed into a *vps6Δ* yeast

strain. Therefore the sole copy of the *VPS6* gene was the one contained on the plasmid. Transformation into a *vps6Δ* yeast strain allowed the expression of the gene carried by the plasmid, without interference from the wildtype (original) copy of the gene in the genome. The transformed yeast were then screened for temperature sensitive alleles. A temperature-sensitive mutant is one that exhibits a wildtype phenotype (no CPY secretion) at a permissive temperature (25°C in this screen), but exhibits a mutant phenotype (CPY secretion) when shifted to the nonpermissive temperature (37°C).

A technique used to study vacuolar protein trafficking in yeast is to assay the amount of CPY secreted. When vacuolar protein trafficking is blocked, CPY is diverted from its original course to the vacuole and is instead secreted at the cell surface. After 9000 colonies were screened, 18 colonies appeared to have the temperature-sensitive behavior, not seen to secrete CPY at the permissive temperature, but seen to secrete CPY heavily at the nonpermissive temperature. Each of the 18 potential mutants was screened three times for secretion and non-secretion at the appropriate temperatures using a CPY overlay assay as seen in Figure 3.

After yeast transformants were identified as being temperature-sensitive for CPY secretion, it was important to determine if the phenotype was dependent on a mutation in the *VPS6* gene on the plasmid rather than a mutation in the yeast genomic DNA. In order to determine if the mutation causing CPY secretion was located on the plasmid, it was necessary to isolate the plasmid out of the yeast cell. After isolation of the plasmid from yeast, it was transformed into *E.coli* where the plasmid was amplified. The plasmid was then removed from the *E.coli* and retransformed into *vps6* Δ yeast cells that had never seen the plasmid. If the temperature-sensitive CPY secretion phenotype

was recapitulated, then the allele had been rescued and the mutation occurred on the plasmid. Of the 18 plasmids recovered, the conditional CPY secretion was observed for 10 plasmids, while the conditional phenotype was lost for 8 others.

Mapping the mutations on the Vps6 protein

To identify where the specific mutations fell within the gene and subsequently, how the mutations affected the sequence of the protein, the DNA was sequenced. DNA is composed of four types of nucleotides, and is read three nucleotides at a time, as a series of codons. A codon identifies the appropriate amino acid to insert when synthesizing a protein. Each of the mutant DNA sequences were compared, nucleotide by nucleotide, to the wildtype *VPS6* sequence. The mutations (errors in the nucleotide sequence) were identified and mapped onto distinct regions of Vps6p.

Lock proteins contain several regions of importance. These regions include the domain where the lock protein recognizes the key protein, as well as a transmembrane domain. A transmembrane domain is the region of the protein that is integrated into the membrane. The regions of Vps6p have been depicted in Figure 4.

Of the 10 temperature sensitive alleles, half contained mutations in the region of the protein immediately prior to the region encoding the transmembrane domain. Figure 5 illustrates the various regions within the Vps6p and correlates a specific mutation found in the mutant sequences to a particular protein region. The mutations, located upstream of the transmembrane domain, have changed a codon within the DNA sequence to a stop codon. A stop codon directs the formation of the protein to stop at its location in the sequence, resulting in a truncated protein. Truncated proteins

(or truncations) are proteins that lack part of the wildtype protein. The location of the stop codons in the sequence suggests, in this case, that the truncated proteins lack a transmembrane domain in contrast to the wildtype protein in Figure 4, and are not physically integrated into the compartment membrane.

The *vps6* temperature sensitive alleles encode truncated proteins

Common sense tells us that if a protein is truncated, then the protein would be of a smaller size. The size of a protein can be determined using a technique called a western blot. In a western blot, a protein is electrophoresed through a polyacrylamide gel. A smaller sized protein is able to fit more easily through the pores in the gel and therefore runs further down through the gel.

To more carefully determine the size of the vps6ts protein in comparison to the wildtype protein, the protein products of *vps6ts* and *VPS6* were mixed and run together on a polyacrylamide gel. Mixing of the protein products from each of the temperature sensitive alleles containing a stop codon prior to the transmembrane domain with Vps6p produced a distinct protein band pattern (Fig.6, lanes 4 and 5). The protein products of the wildtype allele were distinguished by a singlet pattern (lane 1). The singlet pattern and the distance traveled by the proteins on the gel indicated that the upper band seen in the mixed protein band pattern were the result of wildtype protein present in the sample. The two types of proteins, wildtype and truncated Vps6p, differed in size, indicating that the 5 temperature-sensitive mutants that contain a stop codon prior to the transmembrane domain in fact lead to truncations of the Vps6p. With evidence that these 5 vps6ts proteins are truncated and the existence of a stop codon prior to their transmembrane domain, I conclude that the vps6ts mutants lack the transmembrane domain.

Yeast cells expressing truncated form of Vps6p exhibit temperature-sensitive CPY secretion

The most informative *vps6* temperature-sensitive mutants would be those that secrete the lowest level of CPY at the permissive temperature and the highest level after shift to the non-permissive temperature. A second criterion is that the mutant exhibits rapid-onset kinetics, that is CPY secretion occurs shortly after the temperature shift. To identify the rapid onset *vps6ts* mutants, a technique called pulse-chase CPY immunoprecipitation was employed. To do an immunoprecipitation, the newly synthesized CPY is labeled with radioactive cysteine/methionine sulfur-35 and the CPY is tracked, as it is trafficked from compartment to compartment, based on its apparent size as judged by polyacrylamide gel electrophoresis. The modified forms of CPY (p1, pro, and mature) distinguish its extent of travel through the secretory pathway. If the *vps6ts* mutations were to result in a rapid loss of Vps6p function, the block in the vacuolar pathway through the PVC would become apparent quickly after shifting the mutant cells to the nonpermissive temperature.

Figure 7 shows the results of a CPY immunoprecipitation. *vps6Δ* , *VPS6, vps6ts,* and overexpressed *vps6ts* cells were radiolabeled at either 25°C or 37°C for 10 minutes and chased in the presence of excess cold methionine and cysteine for 0 and 60 minutes at the permissive temperature and 0 and 40 minutes at the nonpermissive temperature. The CPY was immunoprecipitated from intracellular and extracellular fractions (see materials and methods). As described in the introduction, the soluble vacuolar hydrolase CPY is first modified in the ER and further modified in the Golgi giving rise to a second form of CPY, or proCPY. CPY is sorted out of the Golgi and transported from there through the PVC to the vacuole and then cleaved to the mature mCPY form

(Bryant and Stevens, 1998). Transport from the Golgi to the PVC is blocked in the temperature-sensitive *vps6ts* cells. Compared with the wildtype yeast, in which CPY was retained intracellularly as mCPY (Fig. 7B, lane 7), the *vps6ts* cells accumulated proCPY within the cell (Fig. 7B, lane 11) and secreted proCPY (lane 12) at the nonpermissive temperature. At the permissive temperature, the mature form of CPY was delivered to the vacuole and processed to the mature form in the temperature-sensitive mutants (Fig. 7A, lane 11). Heavy secretion of proCPY (Fig. 7B, lane 12) - a form of CPY that hasn't seen a proteolytically active compartment such as the PVC - indicates that the transport of CPY from the Golgi to the PVC is defective in these temperature-sensitive mutants at the nonpermissive temperature.

Identification of genes that, when overexpressed, are able to suppress the temperature sensitive phenotype of the truncated allele

As a transmembrane protein, wildtype Vps6p is anchored in the membrane of the PVC. By contrast the truncated Vps6p lacking its transmembrane domain, is free to move throughout the cytoplasm of a cell. Although *vps6Δ* mutant cells containing *vps6ts* secreted CPY at 37^{o}C, when *vps6ts* was overexpressed in *vps6Δ* mutant cells, there was no secretion of CPY at any temperature (Fig. 7A and B, lane 16). To suppress the secretion of CPY, the overexpressed truncated Vps6p may saturate the appropriate Vps6p binding site.

To further test this theory that a binding site exists, the truncated Vps6p was overexpressed by 10 to 20 fold. Consistent with the idea that a binding site exists for mutant Vps6p, overexpression and thus saturation of the binding site eliminates the temperature-sensitive CPY secretion phenotype. If truncated Vps6p achieves its localization through a protein binding site on the PVC, then

increasing the amount of this site should increase the membrane bound fraction of Vps6p that lacks its transmembrane domain. This is the converse of the experiment involving overexpression of the truncated Vps6p. To determine whether the ability of the truncated protein to find its proper location is correlated with the existence of a protein partner to which Vps6p typically binds, a suppression screen using an overexpression library and my *vps6ts* yeast strain was devised. This suppression screen was aimed at identifying proteins that suppressed the temperature-sensitive CPY secretion phenotype by providing the truncated protein, Vps6p, with a greater number of binding sites.

Three possible outcomes may result from this suppression screen. First, I may re-isolate the wildtype gene, *VPS6*, which we know suppresses the effects of the conditional allele. This is important because isolating *VPS6* tells us how well we have screened our library. Each time we recover *VPS6*, this represents screening approximately one genome equivalent of the library. The second possibility is that the gene isolated in the suppression screen affects the localization of the mutant protein, which would allow us to determine proteins necessary for proper docking and fusion of membranes. The third possibility is that a recovered gene encodes for a protein that eliminates or bypasses the requirement for Vps6p.

The suppression screen uncovered 9 suppressors after having screened a library of 9000 colonies. The genes that suppressed the temperature-sensitive phenotype of a truncated protein have all been previously identified as key proteins. The key proteins reside on different compartments within the transport pathway, indicating that the dependence of vesicle fusion and docking may not rely heavily on the specificity of lock and key proteins, but rather the localization of the lock and key proteins, or the specificity of other proteins involved in vesicle fusion and docking.

My first attempt to screen for proteins that suppress the temperature sensitive phenotype is not complete. A few plasmids that suppressed the temperature-sensitive phenotype contained multiple genes and it is unknown which gene is responsible for suppression. I am still in the process of identifying the single gene responsible for the observed suppression. In addition, I failed to retrieve any copies of the wildtype *VPS6* gene. This indicates that an entire genome equivalent has not been screened, and encourages me to continue.

Discussion

In order to study the transport step from the late Golgi to the PVC, I generated several temperature-sensitive alleles of *VPS6*. Of the ten conditional alleles isolated, 5 alleles were rapid onset. These same 5 alleles contained a stop codon directly upstream of the region encoding the carboxy-terminal transmembrane domain. The Vps6 proteins encoded by these 5 alleles lack a transmembrane domain and are therefore not integrated into the target membrane. Although these truncated Vps6 proteins do not span the membrane, they properly sort CPY to the vacuole at the permissive temperature. This result was not predicted, for temperature sensitivity is frequently the result of a mutation that changes the stability of a protein and thereby disrupts important interactions with other proteins, such as that found with lock and key proteins. Instead, all of the rapid onset temperature-sensitive alleles I isolated contain a mutation that removes the transmembrane domain completely and hence disrupts the localization machinery of the Vps6p.

The fact that *vps6ts* produces a protein that is truncated, strongly suggests that the Vps6ts protein is unbound and free to diffuse throughout the cell. CPY is sorted properly at the permissive temperature and even when

vps6ts is overexpressed at the nonpermissive temperature, suggesting that Vps6ts proteins find the appropriate location to bind and function.

For the truncated Vps6p to function properly, I propose that it continues to associate with the membrane of the PVC in an indirect fashion. For a detached Vps6p to find its proper location, one possibility is the presence of an auxiliary protein, separate from Vps6p, yet also bound to the PVC. This auxiliary protein may provide a site to which the truncated Vps6p is able to bind and function.

The idea of an additional protein acting in collaboration with the lock protein modifies the original idea of the lock and key hypothesis that a single membrane protein on both the target and acceptor membranes mediates fusion (Rothman, 1994). This lack of specificity in the lock and key model is not unusual and has been addressed for other lock proteins. As a result of the promiscuity of lock proteins interacting with multiple key proteins, there must be other proteins that account for the specificity of the lock and key complex (Gotte and Fischer von Mollard, 1998). One of these proteins may in fact be the accessory protein needed for truncated Vps6p to localize and function properly.

To find accessory proteins that account for the specificity of this lock and key pair, the suppression screen was designed. In my first screen, I identified 9 suppressors. It is interesting that many of the suppressors have been previously identified as producing key proteins, yet the functional significance is unknown as of yet. It would also be valuable to recover the gene encoding the key protein known to interact specifically with the Vps6 lock protein as well as a copy of the *VPS6* gene itself. For these reasons, I find it encouraging to continue screening the genome for other possible suppressors. After the suppression screen is exhausted, further experiments will be performed to establish the physical relationship of these suppressor proteins with Vps6p.

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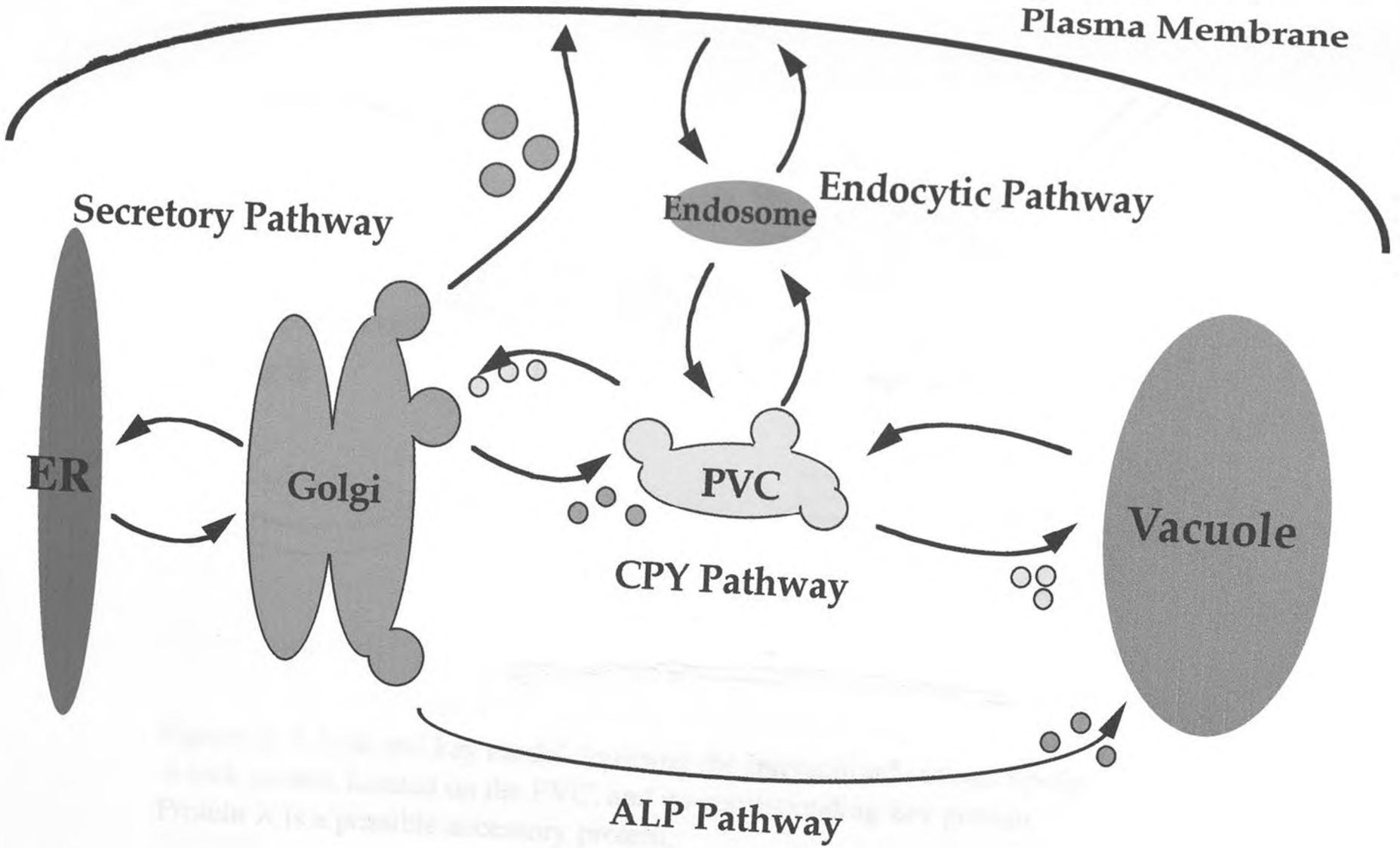

Figure 1. Protein trafficking in yeast

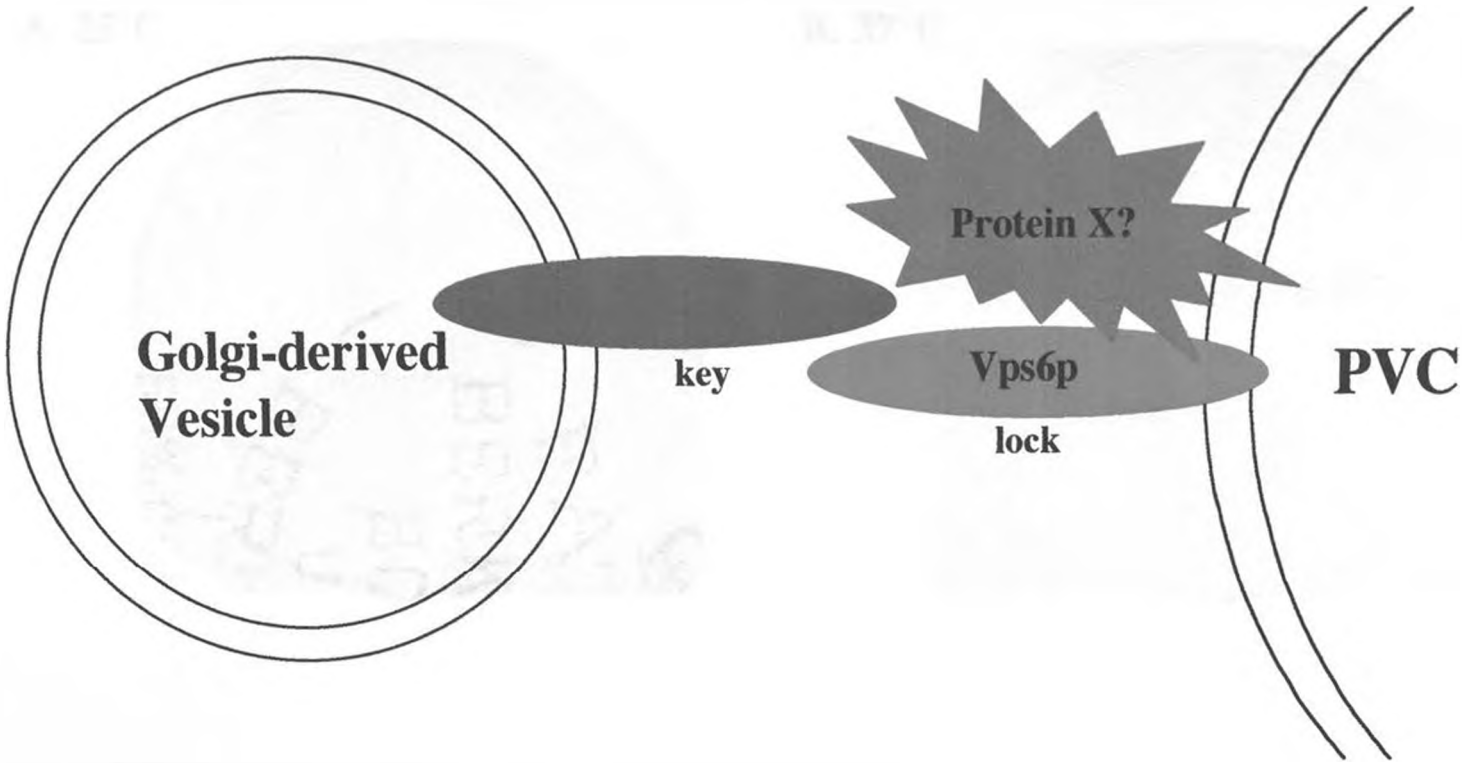

Figure 2. A lock and key model depicting the interaction between Vps6p, a lock protein located on the PVC, and its corresponding key protein. Protein X is a possible accessory protein.

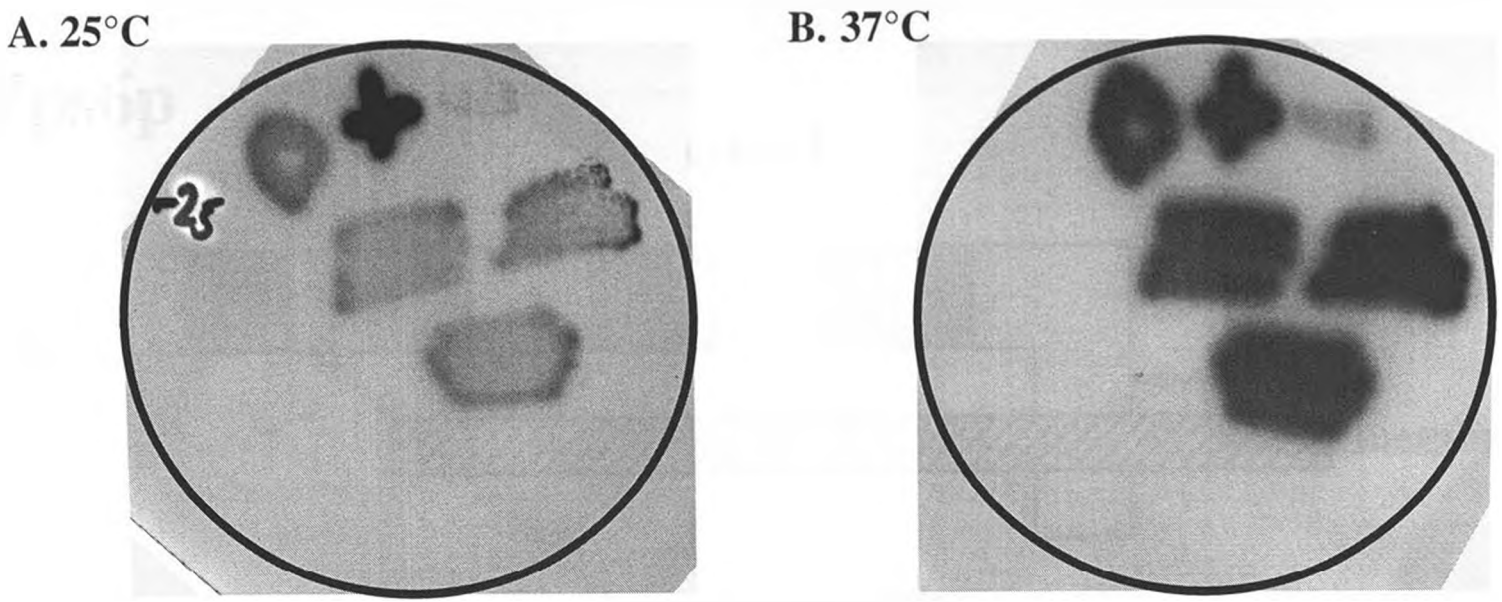

Figure 3. (A) CPY overlay at 25°C. (B) CPY overlay at 37°C. The dark patches represent the secretion of CPY. The "**+**" shows the CPY secretion by the *vps6Δ* strain, while the "**-**" shows the lack of CPY secretion by the *VPS6* strain. The "**o**" depicts the CPY secretion of a previously isolated temperature-sensitive mutant strain, while the patches depict the CPY secretion of 3 potential temperature-sensitive mutants.

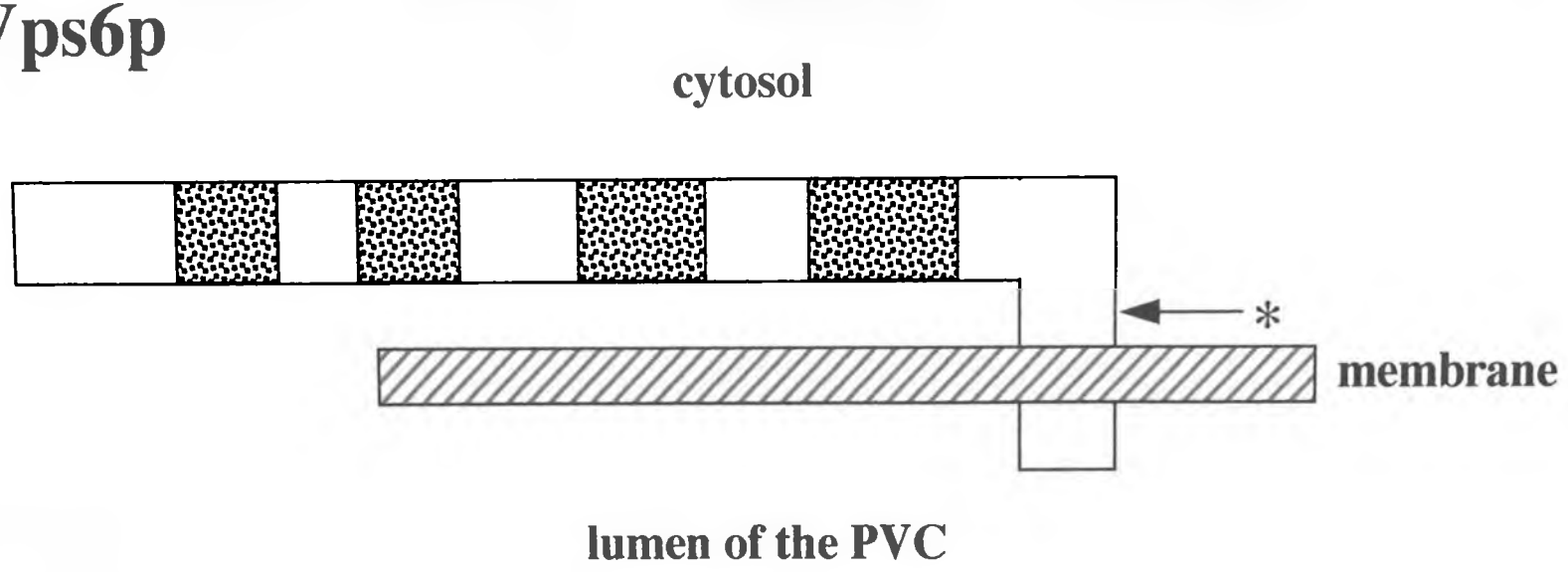

Figure 4. Vps6p integrated into the membrane. Vps6p contains several regions of importance depicted as the speckled boxes on the protein. The temperature sensitive mutants contain a mutation upstream of the region encoding the transmembrane domain (where Vps6p crosses the membrane). The location of the site of truncation is labeled by an asterisk.

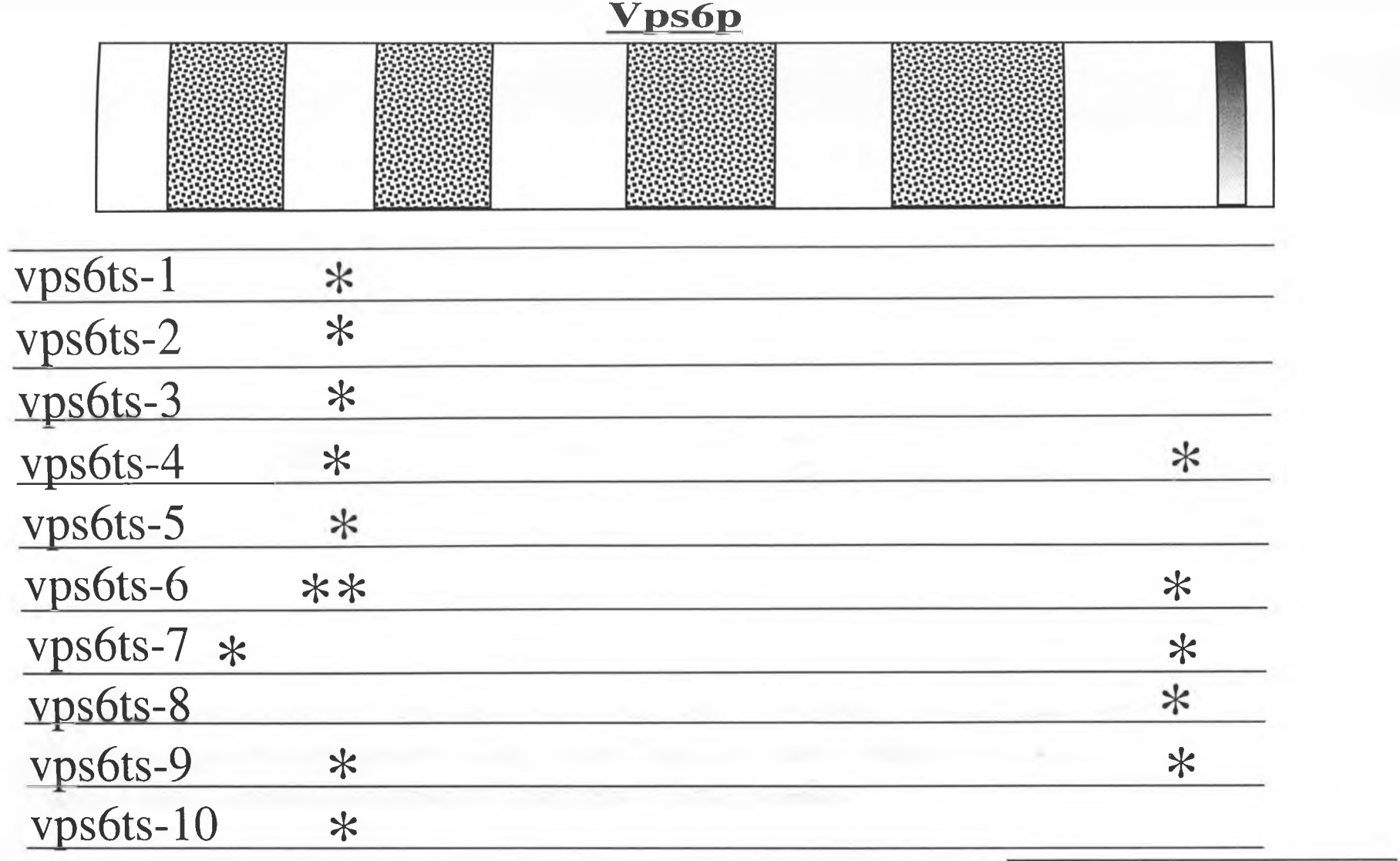

Figure 5. Mapping the sites of codon changes in the Vps6p. The speckled boxes represent regions where Vps6p interacts with other proteins. The transmembrane domain is a shaded region at the far right of the protein. An asterisk represents the site of a codon change corresponding to the region above it.

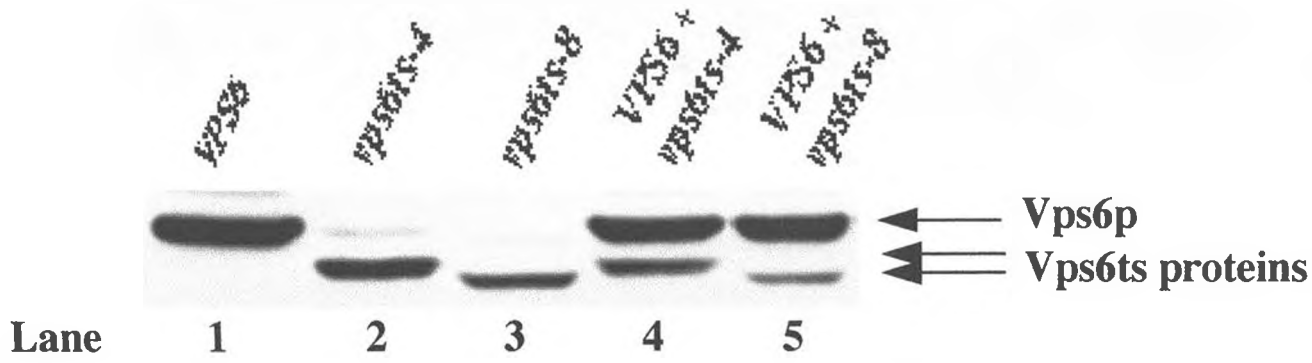

Figure 6. Western Blot of *VPS6* and temperature sensitive alleles, *vps6ts-4* and *vps6ts-8*. The first lane of the gel contains Vps6p, lanes 2 and 3 contain Vps6ts proteins, and lanes 4 and 5 contain a mixture of Vps6p and Vps6ts proteins.

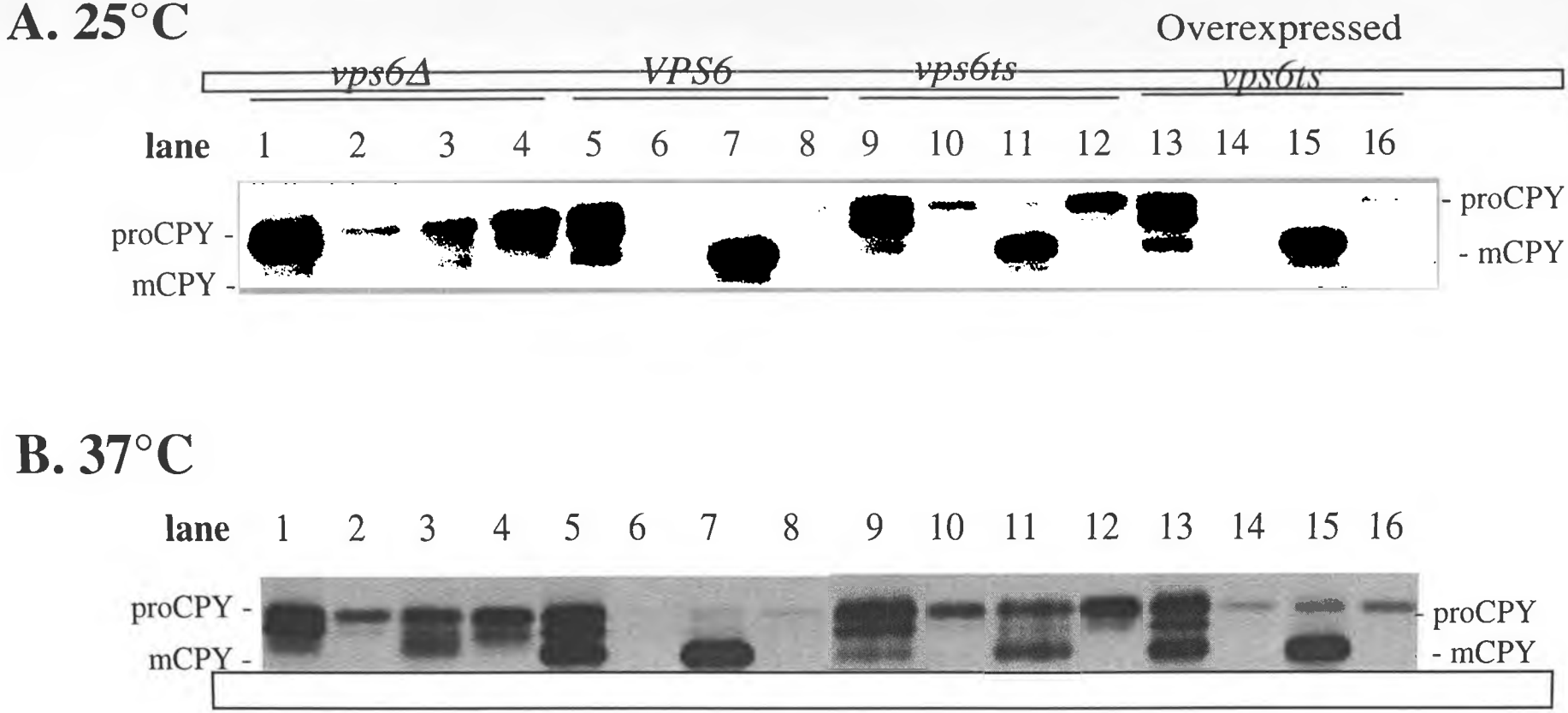

Figure 7. (A) CPY immunoprecipitation of *VPS6, vps6Δ,* and *vps6ts* strains, and also a strain which overexpresses *vps6ts* at 25°C. (B) CPY immunoprecipitation of *VPS6, vps6Δ,* and *vps6ts* strains, and also a strain which overexpresses *vps6ts* at 37°C.