REQUIREMENT OF HSP70s IN THE CYTOSOL TO VACUOLE TRANSPORT OF AMINOPEPTIDASE 1 IN *SACCHAROMYCES CEREVISIAE*

Dissertation

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ABBREVIATIONS:

ADH:	Alcohol dehydrogenase
ALP:	Alkaline phosphatase
AP:	Adaptor complex
ADP:	Adenosine di phosphate
Ape1:	Aminopeptidase 1
pApe1:	Precursor form of Ape1
iApe1:	Intermediate form of Ape1
mApe1:	Mature form of Ape1
APNE:	N-acetyl-phenylalanine-β-naphthyl-ester
APS:	Ammonium per sulfate
ATP:	Adenosine tri phosphate
BSA:	Bovine serum albumin
CE:	Cell extract
cm:	Centi meter
CPS:	Carboxypeptidase S
CPY:	Carboxypeptidase Y
CVT:	Cytosol to vacuole targeting
cAMP:	Cyclic adenosine mono phosphate
°C:	Degree centigrade
dATP	Deoxy adenosine tri phosphate
dCTP	Deoxy cytosine tri phosphate
dGTP	Deoxy guanosine tri phosphate
dTTP	Deoxy thymine tri phosphate
DMSO:	Dimethyl sulfoxide
DNA:	Deoxy ribonucleic acid
DPAP-B:	Dipeptidyl Aminopeptidase B
DTT:	Dithiothreitol
EDTA:	Ethylene diamine tetra aceticacid
ER:	Endoplasmic reticulum
FBPase:	Fructose-1,6-bis phosphatase
Fig:	Figure
g:	Gram
GFP:	green flourescent protein
GTP:	Guanosine tri phosphate
H_2SO_4:	Sulfuric acid
HCl:	Hydochloric acid
HIP:	Hsc70 interacting protein
HOP:	Hsp70/Hsp90 organising protein
HSC:	Heat shock cognate protein
HSP:	Heat shock protein
MHSP70	Mitochondrial HSP70

HRP:	Horse radish peroxidase
KAc:	Potassium acetate
KCl:	Potassium chloride
kDa:	kilo Dalton
kb:	kilo base pairs
LB:	Luria Bertani
LiOAc:	Lithium acetate
LMA1:	Low molecular activity 1
M:	Molar
mA:	Milli ampere
MHC:	Major histocompatibility complex
µg:	Micro gram
mg:	Milli gram
$MgCl_2$:	Magnesium chloride
$MgSO_4$:	Magnesium sulfate
min:	minutes
ml:	Milli litre.
mM:	Milli moles
MPR:	Mannose-6-phosphate receptor
M.W:	Molecular weight
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
ng:	nano gram
nm:	nano meter
NMR:	Nuclear magnetic resonance
NSF:	N-ethylmaleimide sensitive factor
O.D:	Optical density
%:	Percentage
PBS:	Phosphate buffer saline
PCI:	Phenol/ chloroform/ isoamylalcohol
PCR:	Polymerase chain reaction
PEG:	Poly ethylene glycol
pH:	Negative logarithm of the hydrogen ion concentration in moles per litre.
PIPES:	Piperazine-N,N'-bis(2 ethane sulfonic acid)
pmol:	Pico moles
PMSF:	Phenyl methyl sulphonyl flouride
PrA:	Proteinase A
PrB:	Proteinase B
ProK:	Proteinase K
PTS:	Peroxisomal targeting signal
Ref:	Refer
RNA:	Ribonucleic acid
Rpm:	Rounds per minute
S:	Svedberg units

SD-N:	Synthetic media deficient in nitrogen
SDS-PAGE:	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
sec:	secretory mutants
SNAP:	Soluble NSF attachment protein
SNARE:	Soluble NSF attachment protein receptor
t-SNARE:	SNARE on the target membrane
v-SNARE:	SNARE on the vesicle membrane
Sq.cm:	Square centimeter
TBS:	Tris buffered saline
TBST:	Tris buffered saline tween
TCA:	Trichloroacetic acid
TE:	Tris EDTA buffer
TEMED:	N'N'N'N tetramethylethylenediamine
TGN:	Trans Golgi network
Tlg:	t-SNARE of the late Golgi
ts:	Temperature sensitive
Ubc:	Ubiquitin conjugating enzyme
UV:	Ultra violet
V:	Volts
vps:	Vacuolar protein sorting
WT:	Wild type
w/v:	Weight/ Volume
YPD:	Yeast extract Peptone Dextrose

1. INTRODUCTION

Saccharomyces cerevisiae is a single celled eukaryote. It contains all the organelles typical of eukaryotes namely the nucleus, mitochondria, endoplasmic reticulum, Golgi complex and the vacuole. Proteins, after synthesis, are transported into these organelles where they perform various functions thus maintaining the functional integrity of the cell. Heat shock proteins of the 70 kDa family are known to assist in the transport of proteins into the nucleus, mitochondria and ER.

1.1 The yeast vacuole

The yeast vacuole is similar to lysosomes in animal cells and vacuoles in plant cells. The vacuole occupies about 10-20 % of the total cell volume in exponentially growing cells, in nutrient rich media. However, the size varies based on the growth phase and conditions and it can reach a size of upto 80 % of the cell volume. The lumen of the vacuole is acidic and it contains a variety of hydrolytic enzymes. Vacuoles are required in the cell for maintaining pH and osmotic homeostasis (Klionsky *et al*., 1990; Nelson and Nelson., 1990; Yamashiro *et al*., 1990; Anraku *et al*., 1989; Banta *et al.,* 1988). The vacuole is a major reserve for basic amino acids, inorganic phosphate and calcium ions. These reserves are mobilized in response to nutrient limitations. Vacuolar enzymes release amino acids from intracellular proteins (protein turn over) in response to nutrient limitation (Zubenko and Jones, 1981). The vacuole is thus known to participate in several important physiological functions in response to changing growth condition.

Proteinase A and proteinase B are two vacuolar resident endopeptidases responsible for the activation of other proteases in the vacuole (Van De Hazel *et*

al., 1992). Proteinase A is an aspartyl protease, homologous to the mammalian cathepsin D. Proteinase B is a serine protease homologous to the bacterial subtilisin (Moehle *et al*., 1989). *PEP 4* gene encodes for proteinase A which is the 'master switch' for the activation of a variety of other vacuolar proteases. The Golgi form of the protein (52 kDa) is converted into the 42 kDa active form autocatalytically (Wolf *et al*., 1996). Cells lacking proteinase A do not survive conditions of nutrient starvation. This might be because of its role in processing a number of vacuolar enzymes to their mature form. Proteinase B can not substitute for proteinase A. Protein degradation is retarded in ProB deficient cells during starvation, but in contrast to proteinase A deficient cells they do not die under these conditions.

1.2 Delivery of proteins into the vacuole

1.2.1 Transport of soluble proteins into the vacuole

Soluble proteins are transported into the vacuole along four pathways namely, secretory pathway, cytosol to vacuole targeting pathway, autophagocytosis and endocytosis. Vacuolar resident proteins are transported along the secretory and cytosol to vacuole targeting pathway. Proteins destined for degradation are transported by autophagocytosis and endocytosis.

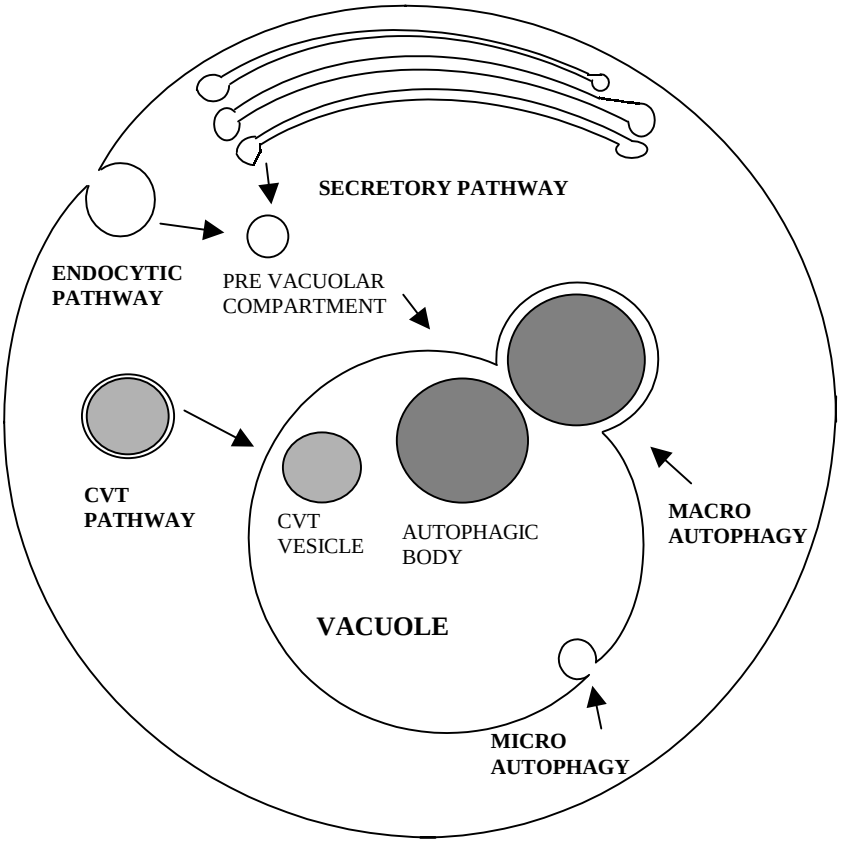


Fig1: The different pathways transporting proteins into the vacuole in the yeast *Saccharomyces cerevisiae.*

(i) Vacuolar entry along the secretory pathway

Majority of resident vacuolar proteins enter the vacuole by this pathway. The proteins translocate across the ER membrane via an amino terminal cleavable signal or a non-cleaved internal hydrophobic signal (Rapoport *et al*., 1996). Cleavage of the signal sequence releases the protein into the lumen of the ER, in case of secretory proteins. Vacuolar hydrolases are glycosylated in the ER. Glycosylation appears to be required for the efficient sorting of proteinase A however, it does not serve as a targeting signal for CPY (Winther,

1989). Delivery to the vacuole is dependent on a second targeting signal present in the 'pro' region of the precursor. On reaching the vacuole these proteins are processed into their mature form in a proteinase A and proteinase B dependent manner (Mechler *et al*., 1987). Carboxypeptidase Y (CPY) is a well characterised soluble vacuolar hydrolase which enters the vacuole by this pathway (Raymond *et al*., 1992a). Over expression of CPY leads to its missorting and secretion (Rothman and Stevens, 1986; Stevens *et al*., 1986). The secreted CPY is in its Golgi derived form. The secretion of CPY is blocked in *sec1* ts mutants at non permissive temperatures indicating that the precursor form of CPY transits through the late secretory pathway. Similarly over expression of proteinase A (another soluble vacuolar glycoprotein) also results in the secretion of the protein. Such a missorting of the proteins due to over expression could be because of the presence of a saturable receptor (Stevens *et al*., 1986). Current data supports a model in which a receptor, Vps10p, recognises its ligand, in the late Golgi. The receptor ligand complex leaves the Golgi apparatus and is delivered to a pre vacuolar compartment where the receptor dissociates from the ligand and recycles back to the Golgi apparatus. Vps10p is required for the sorting of both CPY and proteinase A (Cooper and Stevens, 1996). Homologs of Vps10p have been found in the *S. cerevisiae* genome. There is evidence that one of the homologs Vth2p (Vps Ten Homolog) can act as a functional receptor for CPY and PrA. These data suggest that there is a family of receptors, in *S. cerevisiae*, that participate to various degrees in the sorting of soluble hydrolases to the vacuole. However, these receptors are expressed at much lower levels than Vps10p (Cooper and Stevens, 1996; Westphal *et al*., 1996). The transport of CPY and PrA by Vps10p to the vacuole is reminiscent of the mannose-6-phosphate receptor (MPR) dependent transport of lysosomal enzymes in mammalian cells (Pfeffer, 1988; Braulke *et al*., 1987).

(ii) Cytosol to Vacuole Targeting Pathway (CVT Pathway)

Two vacuolar resident enzymes α- mannosidase and aminopeptidase 1 (Ape1) are transported to the vacuole by the cvt pathway.

There are four identified aminopeptidases in *S. cerevisiae* that hydrolyse leucine substrates (Trumbly and Bradley, 1983). Based on subcellular fractionation experiments it appears that only Ape1p is localised to the vacuole (Frey and Röhm, 1978; Matile *et al*., 1971). The *APE1* gene encodes for a protein of 514 amino acids containing four potential sites for N-linked glycosylation (Chang and Smith, 1989; Cueva *et al*., 1989). The active enzyme is a multimeric protein having a molecular mass of approximately 600 kDa. The mature Ape1p monomer has a molecular mass of 50-51 kDa based on the migration on a denaturing gel (Chang and Smith, 1989; Metz and Röhm, 1976). The molecular mass based on the amino acid composition predicts a protein of approximately 45 kDa (Metz and Röhm, 1976). It has been shown that Ape1p is synthesized as an inactive zymogen that is processed in a *PEP4* dependent manner (Chang and Smith, 1989; Cueva *et al.*, 1989; Trumbly and Bradley, 1983) like all other soluble vacuolar proteins that are transported along the secretory pathway. The maturation of Ape1p from its precursor form has been shown to be a two step process. The 61 kDa precursor is converted into a 55 kDa intermediate and then into the 50 kDa mature, active form. Using mutants defective in proteinase A (encoded by *PEP4)* and proteinase B activity it has been shown that the conversion of the precursor to the intermediate form is a result of proteinase A activity and the conversion of the intermediate to the mature form is brought about by proteinase B (Seguí-Real *et al*., 1995).

The following observations led to the conclusion that Ape1p does not traverse the classical secretory pathway-

(i) Using tunicamycin, which blocks the addition of N-linked oligosaccharides to proteins, and Concanavalin A, to which both the N-linked

and O-linked oligosaccharides bind, it was shown that aminopeptidase 1 is not glycosylated, even though Ape1p has four potential glycosylation sites (Klionsky *et al*., 1992).

(ii) Ape1p has a half time of transport of 45 min when compared to a half time of approximately 6 min for CPY, proteinase A or alkaline phosphatase (ALP) (Klionsky and Emr, 1989; Klionsky *et al*., 1988; Hasilik and Tanner, 1978).

(iii) Ape1p does not contain the standard ER signal sequence. Using various temperature sensitive *sec* mutants, defective in the secretory pathway, it was shown that Ape1p does not enter the secretory pathway.

(iv) Over production of CPY and PrA leads to their secretion but over expressed Ape1p is not secreted (Klionsky *et al.*, 1992).

These observations led to the conclusion that Ape1p is transported directly from the cytosol into the vacuole along the cytosol to vacuole targeting pathway.

Ape1p contains a 45 amino acid N-terminal pro peptide region. The pro peptide has two α-helices separated by a β-turn. Physical and chemical properties of the pre sequence of pApe1p have been analysed to determine their relevance in the transport from cytosol to vacuole. The pre sequence, containing 17 residues, folds to distinct amphipathic helices in aqueous and hydrophobic environments. Using NMR spectroscopy it has been shown that mutations which destabilise the first α-helix inhibit the transport of Ape1p (Martinez *et al*., 1997). Ape1p targeting is sensitive to changes in periodicity and content of the predicted amphipathic α-helix but insensitive to similar changes in the second helix (Oda *et al*., 1996).

It has been shown that the prepro part of Ape1p is necessary and sufficient to target the protein into the vacuole. This was shown by tagging GFP to the prepro part of Ape1p. This construct was targeted into the vacuole and

the reporter protein was released. They have also shown that the separated pre and pro parts do not target GFP to the vacuole (Martinez *et al*., 1999).

The following mechanisms have been suggested for the transport of Ape1p into the vacuole-

(i) Translocation: The transport of a protein along a proteinaceous channel in the organellar membrane is called translocation.

Ape1p with a `myc' tag attached to its `C' terminus was found to stick across a membrane. The myc tag was accessible to externally added proteases. The N-terminal signal sequence was cleaved off. This cleavage is known to be dependent on PrA and PrB found in the vacuole. This indicated that the protein was stuck across the vacuolar membrane. Hence it was suggested that the transport of Ape1p is by translocation (Seguí-Real *et al*., 1995). This appears to be similar to the transport of proteins into the ER and mitochondria (Schatz and Dobberstein, 1996)

(ii) Vesicle mediated process: Studies with Ape1p have shown that it enters the vacuole as a dodecamer. Pulse chase experiments suggested that oligomerisation is an early event in transport (Kim *et al.*, 1997). The half time for transport of Ape1p into the vacuole is about 45 min while the half time of oligomerisation is about 2 min. Mutants in the cvt pathway or propeptide deletion mutants are shown to be defective only in the import step and not in oligomerisation. The import into the vacuole appears to be the rate limiting step (Kim *et al*., 1997). This transport of a large oligomer into the vacuole appears to be similar to the transport of large pre assembled oligomeric complexes into the peroxisomal lumen (Rachubinski and Subramani, 1995; Subramani, 1993). It has been suggested that the oligomeric precursor Ape1p is enwrapped by a double membrane (Baba *et al*., 1997, Scott *et al*., 1997) and this cvt vesicle transports Ape1p into the vacuole.

It has also been suggested that the mechanism by which the cvt vesicle is transported to the vacuole is similar to autophagy (Baba *et al*., 1997). Most of the isolated autophagy mutants are known to be defective in the cvt pathway also. Both the cvt and autophagic vesicles are double membrane vesicles. However both the cvt and autophagy are two distinct pathways. Ape1p transport occurs constitutively in the cell while autophagy takes place only under nutrient limitation. The cvt vesicles are smaller (150 nm in diameter) compared to the autophagosomes (300-900 nm in diameter).

(iii) Autophagocytosis

Autophagy is defined as the process of bulk degradation of cytoplasmic proteins or organelles in the lytic compartment (Dunn, 1994; Mortimore *et al*., 1989; Schworer *et al*., 1981). Vacuolar proteolytic activity increases under conditions of nitrogen starvation. 45 % of all the cellular proteins are degraded in the vacuole within 24 hours (Teichert *et al*., 1989). The autophagic pathway is non specific and this was shown by following the concomitant uptake of several cytosolic proteins into the vacuole under starvation conditions (Egner *et al*., 1993). In the absence of proteinase machinery and under starvation conditions vesicles are seen accumulating in the vacuoles (Takeshige *et al*., 1992).

Autophagy can be of two types, macroautophagy and microautophagy. Macroautophagy is the major route under nutrient starvation conditions. Double membrane structures are formed in the cytosol and these engulf the cytosolic proteins and organelles. These double membrane vesicles are called `autophagosomes'. The number of autophagosomes per cell is quite low suggesting that it is a transient intermediate structure that fuses with the vacuole rapidly after formation (Klionsky and Ohsumi, 1999). The size of yeast

autophagosomes ranges from 300 to 900 nm in diameter, which is similar to the size of mammalian autophagosomes (Baba *et al.*, 1994). The autophagosomes fuse with the vacuole and release a unilamellar vesicle inside the lumen. This vesicle is subsequently degraded in a proteinase B dependent manner (Takeshige *et al*., 1992).

Microautophagy is defined as a process of incorporation of cytoplasmic components by invagination or engulfment of the vacuolar/ lysosomal membrane (Dunn, 1994; Mortimore *et al*., 1988; Ahlberg and Glaumann, 1985).

There are mainly two sets of autophagy mutants that have been isolated, the *apg* and *aut* mutants. Cells defective in autophagy become sensitive to starvation conditions. The *apg* (autophagy) mutants were screened based on this and the morphological criteria. The first *apg* mutant was, however, isolated based on the accumulation of autophagic bodies within the vacuole (Tsukada and Ohsumi, 1993).

The cytosolic enzyme fatty acid synthase is degraded in the vacuole in a *PEP4* dependent manner. Autophagy mutants, *aut*, which accumulated fatty acid synthase in the vacuole under starvation conditions were isolated. A second set of mutants which failed to accumulate the enzyme in the vacuole were also isolated (Thumm *et al*., 1994).

A third set of mutants were isolated based on an alkaline phosphatase assay system (Noda *et al*., 1995). Cells were constructed with a modified version of the vacuolar membrane protein alkaline phosphatase (encoded by the *Pho8* gene). This gene lacked the sequences for the N-terminal 60 amino acids. This truncated alkaline phosphatase (Pho8Δ60p) was unable to enter the vacuole along the secretory pathway, as the non mutated form. The protein was expressed in the cytosol and entered the vacuole by autophagy under starvation conditions. This truncated form on entry into the vacuole was processed into its active form. So the appearance of alkaline phosphatase activity was an

indication for the nonselective uptake of proteins. Several mutants were obtained by this screen but have not been further characterised (Klionsky and Ohsumi, 1999).

Yeast cells induce sporulation under nitrogen starvation conditions. So the cell differentiation event proceeds via the degradation of pre existing macromolecules and organelles. Other than nitrogen deficiency, carbon, sulphate, phosphate and auxotrophic amino acid starvation also induces autophagy to various extents (Takeshige *et al.*, 1992). No other stress conditions have been reported to induce autophagy. Induction of autophagy is regulated by a phosphatidyl inositol kinase homolog called `TOR' (Noda and Ohsumi, 1998). Tor prevents the induction of autophagy when the cells are in nutrient rich media. Rapamycin is an inhibitor of Tor kinase and it induces autophagy in nutrient rich media. High levels of cAMP is inhibitory for autophagy (Noda and Ohsumi, 1998).

Cells have the ability to degrade proteins which are no longer necessary. The gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) is an example for this. FBPase is induced during glucose starvation. On addition of glucose FBPase is targeted for degradation. This has been called catabolite induced inactivation (Purwin *et al.*, 1986; Pohlig and Holzer., 1985; Mazon *et al.*, 1982).

FBPase contains three potential glycosylation sites, however the protein is not glycosylated. Sec18p (NSF homolog in yeast) is required for the vesicle mediated transport steps and not the translocation of proteins. In a *sec18* ts mutant, at the restrictive temperature, translocation into the ER would take place normally but then further vesicle mediated transport would be affected. As a result proteins being transported along the secretory pathway would accumulate in the ER. Using *sec18* ts mutants it was shown that the protein accumulated in the cytosol, after the cells were transferred to the restrictive temperature, and not in the ER. Hence it was proposed that FBPase is

transported directly from the cytosol to the vacuole for degradation. It has also been suggested that the protein is imported into vesicles (about 30-40 nm in diameter) before entry into the vacuole (Huang and Chiang, 1997).

However ubiquitination has been shown to be a prerequisite for the degradation of FBPase. It has been suggested that the polyubiquitinated enzyme is then degraded by the 26S proteasome machinery (Schork *et al.*, 1995). Recently a ubiquitin conjugating enzyme Ubc8p has been shown to be involved in the glucose induced ubiquitination of FBPase. This has been shown to be a prerequisite for the catabolite induced inactivation of FBPase. Ubc8p and FBPase have been shown to be present in the cytoplasmic fraction of the cell (Schule *et al*., 2000).

(iv)Vacuolar entry via endocytosis

Haploid yeast cells are one of the two mating types ‘a’ or ‘α’. Conjugation between these two mating types leads to an a/α diploid cell. Mating is initiated in response to a peptide pheromone stimulus that channels through specific receptors. Cells of the ‘α’ mating type secrete the ‘α’ factor which binds to the ‘α’ factor receptor (encoded by the *STE 2* gene) on the cells of the ‘a’ mating type. Similarly, cells of the ‘a’ mating type secrete the ‘a’ factor which binds to the receptor on the cells of the opposite mating type (α mating type). The pheromone bound receptor is endocytosed and degraded in a *PEP4* dependent manner (Singer and Riezmann, 1990; Dulic and Riezmann, 1989; Konopka *et al*., 1988; Chvatchko *et al*., 1986).

1.2.2 Delivery of proteins to the vacuolar membrane

In *S. cerevisiae* two type II integral membrane proteins, alkaline phosphatase (ALP) and dipeptidyl aminopeptidase B (DPAP-B) have been studied for determining the sorting of proteins to the vacuolar membrane. Till recently it was believed that the transport of both ALP and DPAP-B to the vacuole is by default (Kelly, 1985). *Vps* mutants are defective in vacuolar protein sorting. Vps45p is required for fusion of the Golgi derived vesicles with the pre vacuolar compartment (pvc), (Conibear and Stevens, 1995; Horazdovsky *et al*., 1995) and Vps27p is required for the transit of proteins out of the pvc into the vacuole (Piper *et al*., 1995; Raymond *et al*., 1992b). Using *vps45*-ts and *vps27*-ts mutants it has been suggested that ALP enters the vacuole directly from the Golgi and does not enter the transport vesicles destined for the vps pathway or those destined for the plasma membrane. A sorting determinant in the cytosolic tail of ALP has been characterized. The necessary determinants of the signal were found in the amino terminal 16 amino acid portion of the cytosolic domain (Piper *et al*., 1997). Further, in *vps41* mutants the transport of CPY to the vacuoles was not affected whereas the transport of ALP was disrupted indicating the existence of a novel Golgi to vacuole delivery pathway in yeast (Cowles *et al*., 1997a).

Adaptor complexes (AP) are known to function in the formation and budding of transport vesicles by recruiting coat proteins to the donor membrane. AP-1 functions at the TGN and AP-2 at the plasma membrane. AP-1 and AP-2 both recruit clathrin while AP-3 does not bind to clathrin (Simpson *et al*., 1996, Newman *et al*., 1995). In mammalian cells AP-3 has been localised to late Golgi and endosomal membrane (Dell'Angelica *et al*., 1997; Simpson *et al*., 1997). It has been shown that AP-3 complex is involved in the transport of ALP to the vacuole. ALP and the vacuolar t-SNARE Vam3p are both mislocalised in AP-3 deletion strains. However, transport of the other vacuolar proteins like CPY and

CPS (carboxypeptidase S) is not affected in these mutants. It is also shown that sorting of ALP and other proteins to the vacuole is not affected in AP-1 and AP-2 mutants. ALP was found to accumulate in non vacuolar membrane bound vesicles and tubules in the AP-3 mutants. This was shown using cells expressing GFP tagged ALP (Cowles *et al*., 1997b).

1.3 Heat Shock Proteins of the 70 kDa family

Heat shock proteins (HSP) are synthesized in all organisms in response to environmental stresses, like an increase in temperature. This group of proteins contains various members like the HSP70s, HSP60s, HSP90s etc. Heat shock proteins of the 70 kDa family, HSP70s, are highly conserved from bacteria to man (Boorstein *et al*., 1994; Lindquist and Craig, 1988). The amino acid identity between the HSP70 members from prokaryotes to eukaryotes is about 50 %. Members of this family are not only synthesized under heat shock conditions, as the name suggests, but are also constitutively expressed (Boorstein *et al.,* 1994; Gething and Sambrook, 1992; Lindquist and Craig, 1988; Lindquist, 1986; Craig and Jacobsen, 1985). HSP70s are expressed in almost all cellular compartments including nuclei, mitochondria, chloroplasts, ER and cytosol.

1.3.1 Functions of HSP70s in the cell:

HSP70s are known to perform various functions in the cell-

1. They prevent the accumulation of denatured proteins generated as a result of exposure to high temperature or some other stress (Parsell *et al*., 1993). Interaction between various peptides and the constitutively expressed cytosolic HSP70 and the ER lumenal HSP70, BiP, were studied. Based on these studies it was proposed that a hinderance in the steric accessibility of peptide segments is what prevents the interaction between the HSP70s and folded proteins (Flynn *et al.,* 1989).

2. They bind to the nascent polypeptide chain on the ribosome thus preventing misfolding of the protein (Beckmann *et al*., 1990).

3. HSP70s are involved in the targeting of proteins to their correct destinations. Cytosolic HSP70s are known to assist in the translocation of proteins across the ER (Rapoport *et al*., 1996; Caplan *et al*., 1992a; Chirico *et al*., 1988; Deshaies *et al*., 1988) and mitochondrial membrane (Hachiya *et al*., 1995). They are also known to bind to the KFERQ sequence of cytoplasmic proteins bound to the lysosomes for degradation (Terlecky *et al*., 1992; Dice *et al*., 1990; Chiang *et al*., 1989, 1988).

4. HSP70s present within various organelles assist in protein transport across the organellar membrane-

(a) Cellular depletion of the ER lumenal HSP70, Kar2p, leads to cytosolic accumulation of the ER precursor proteins in *S. cerevisiae* (Nguyen *et al*., 1991; Vogel *et al*., 1990). Recently using *KAR2* alleles, the involvement of Kar2p in retrograde transport from the ER to the cytosol has been shown. A mutant form of CPY was observed to be accumulated in the ER lumen of these mutants while its import was not affected. This showed that Kar2p might interact with the translocation machinery for the retrograde transport of the mutant proteins from the ER to the cytosol or it might be involved in the unfolding of the mutant protein before export (Plemper *et al*., 1997).

(b) In *S. cerevisiae* the mitochondrial matrix contains an Hsp70 termed MHSP70 (mitochondrial HSP70). MHSP70 plays a bipartite role. It assists in the translocation of the unfolded proteins from the cytosol across the mitochondrial membrane and in the folding of these proteins in the matrix (Satyanarayana, C. and Horst, M. 1998; Horst *et al*, 1997; Kronidou *et al*., 1994).

(c) In the inter membrane space of chloroplasts an HSP70 like protein is anchored to the inner face of the outer membrane (Schnell *et al*., 1994; Marshall *et al*., 1990). It might be involved in the transport of proteins across the outer membrane into the chloroplast.

5. Uncoating ATPase is a protein which assists in releasing the clathrin triskelion from clathrin coated pits. The uncoating ATPase is shown to be a member of the HSP70 family (Chappel *et al.,* 1986). The uncoating ATPase is able to recognise a conformation when the arms of the triskelion are juxtaposed in an anti parallel manner. This conformation triggers the ATPase activity of the uncoating protein and the energy generated by the ATP hydrolysis leads to the disassembly of the clathrin (Chappel *et al.,* 1986). Recently it has been suggested that HSC70 not only uncoats clathrin but also prevents it from inappropriate polymerisation in the cytosol. In addition it is also proposed that HSC70 primes the released clathrin to form coated pits again (Jiang *et al*., 2000).

1.3.2 Structure of HSP70s:

HSP70s have two distinct domains, the N-terminal and C-terminal domain. The 44 kDa N-terminal domain binds nucleotides and this is called the ATPase domain (McKay., 1991; Flaherty *et al.,* 1990). This domain is highly conserved. X-ray crystallography studies have shown that the N-terminal domain consists of a bilobed structure with a deep cleft in which nucleotides bind (Flaherty *et al.,* 1990). Many of the highly conserved residues of the N-terminal domain lie in the ATP binding cleft. The crystal structure of bovine HSC70 has been determined. It has a four domain structure with similarities to actin and hexokinase (Flaherty *et al*., 1990).

The 28 kDa C-terminal domain is less conserved and interacts with the substrate proteins and polypeptides (Boorstein *et al*., 1994; Fourie *et al*., 1994). Based on the slight similarities in primary sequence and secondary structure predictions it is proposed that the C-terminal domain is similar to the MHC

class I antigen presenting molecule (Flajnik *et al*., 1991; Rippmann *et al*., 1991).

Structural analysis of the substrate binding unit of the bacterial HSP70, DnaK, has shown that it consists of two halves. The first half is a compact β sandwich followed by an extended α helix. The second half consists of five α helices. The peptide is shown to bind to the β sandwich while the α helices appear to function as a lid that encapsulates the substrate. On the basis of this structural analysis it is suggested that in the ADP bound state, when the substrate is tightly bound, HSP70 molecule has the α helical lid closed while in the ATP bound state, when the substrate is released, the α helical lid is displaced (Zhu *et al*., 1996).

The structure of HSP70s suggests that they share a common mechanism for utilising the energy of ATP and interacting with various substrates (Parsell *et al*., 1993). The two domains are interdependent, the intrinsic ATPase activity of the HSP70 is stimulated by peptide binding and release of the peptide substrate also depends on the nature of the nucleotide bound. Nucleotide binding, substrate binding and phosphorylation alter the conformation of HSP70s (Blond-Elguindi *et al*., 1993; Gaut and Hendershot, 1993; Palleros *et al*., 1993; Toledo *et al*., 1993; Gething and Sambrook, 1992; Hartl *et al*., 1992). Substrates bind to HSP70s when it is in the ATP bound state. The interaction between the substrate and the ATP bound HSP70 is weak and as a result the bound substrate can be released. So in the ATP bound state there is an equal possibility of substrate binding and release. The ATPase activity, of HSP70, leads to a stable interaction between the ADP-HSP70 and the already bound substrate (Greene *et al*., 1995; McCarty *et al*., 1995; Schmid *et al*., 1994; Palleros *et al*., 1993) because of which there is a low possibility of the substrate being released. ATP binding to the ATPase domain causes a conformational change which leads to a structural alteration in the C-terminal domain resulting in substrate release (Buchberger *et al*., 1995; Freeman *et al*., 1995; Schmid *et*

al., 1994; Palleros *et al*., 1993; Liberek *et al*., 1991). During heat shock conditions proteins are partially unfolded. In this unfolded state the hydrophobic surfaces are exposed which then interact and form aggregates. It is suggested that HSP70s bind tightly to the exposed hydrophobic surfaces and restrict their interaction. HSP70 is then released from the substrate due to a conformational change as a result of ATP binding. The released substrate could now reassemble or refold into its correct conformation. Repeated cycles of binding and release from HSP70 would result in the repair of denatured proteins (Pelham, 1986). It is also suggested that HSP70s function in a similar manner to assist the folding and assembly of proteins which are being synthesized.

1.3.3 Interacting partners of HSP70s

The intrinsic ATPase activity of HSP70s is very low, as a result the ATP bound form of the protein with low substrate affinity is predominant (Greene *et al*., 1995; McCarty *et al*., 1995; Schmid *et al*., 1994; Palleros *et al*., 1993). The chaperone activity of HSP70 is regulated by factors that assist the inter-conversion between ATP and ADP bound states. The DnaJ and GrpE proteins of *E. coli* are known to perform such a function (McCarty *et al*., 1995; Wawrzynow *et al*., 1995; Szabo *et al*., 1994; Hendrick *et al*., 1993; Schröder *et al*., 1993; Langer *et al*., 1992; Liberek *et al*., 1991). DnaJ contains a characteristic `J' domain which has been conserved in all the DnaJ like proteins found in eukaryotes. DnaJ increases the ATPase activity of DnaK while GrpE promotes the nucleotide exchange (McCarty *et al*., 1995; Szabo *et al*., 1994; Liberek *et al*., 1991). Concerted action of DnaJ and GrpE increases the ATPase activity of DnaK by up to about 50 fold or more (Liberek *et al*., 1991). DnaJ has the ability to bind to unfolded polypeptides (Szabo *et al*., 1994; Hendrick *et al*., 1993; Schröder *et al*., 1993; Langer *et al*., 1992) and target the substrate to

DnaK. This results in the formation of a ternary complex of DnaJ, DnaK bound to ADP and the substrate (Szabo *et al*., 1994; Langer *et al*., 1992). GrpE catalysed ADP-ATP exchange dissociates the complex. The substrate is now free and might fold, be transferred to another chaperone system or could bind back to DnaJ and DnaK (Hendrick and Hartl, 1995).

DnaJ and GrpE homologs have been found which cooperate with the HSP70s in the eukaryotic cytosol and in the lumenal spaces of mitochondria and ER (Bolliger *et al*., 1994; Ikeda *et al*., 1994; Laloraya *et al*., 1994; Rowley *et al*., 1994).

Ydj1p and Sis1p are DnaJ homologs found in the cytosol of *S. cerevisiae*. Ydj1p is farnesylated and associated with the cytosolic face of the ER and nuclear membrane (Caplan *et al*., 1992b). Ydj1p has been shown to interact with Ssa1p, one of the yeast cytosolic HSP70s, by stimulating its ATPase activity and thus influencing its interaction with the substrates (Ziegelhoffer *et al*., 1995; Cyr *et al*., 1992). *ydj1* mutants have a defect in the import of F1β into the mitochondria (Atencio and Yaffe, 1992; Caplan *et al*., 1992a) and the preproαfactor into the ER (Caplan *et al*., 1992a) implying the involvement of Ydj1p along with the cytosolic HSP70s in protein transport to the different organelles.

Sis1p interacts with the Ssb proteins. Temperature sensitive mutants of Sis1p are defective in the initiation of translation. Sis1p is mainly associated with the 40S subunits and polysomes (Zhong and Arndt, 1993).

Sec63p, a DnaJ homolog is present in the ER membrane of *S. cerevisiae* and in association with the ER lumenal HSP70, Kar2p, it is involved in protein translocation into the ER (Rothblatt *et al*., 1989).

Mdj1p is the DnaJ homolog in the mitochondrial matrix. It stimulates the ATPase activity of MHSP70 indicating its role during protein folding (Horst *et al*., 1997; Rowley *et al*., 1994). Along with the MHSP70 it is involved in the degradation of misfolded proteins and protection of proteins against heat

induced aggregation (Prip-Buus *et al*., 1996; Westermann *et al*., 1996; Rowley *et al*., 1994; Wagner *et al*., 1994).

HSP40 (Hdj1p) is the human homolog of DnaJ. It interacts with the constitutively expressed HSP70 (HSC70) in the mammalian cytosol. HSP40 stimulates the ATPase activity of HSC70 thus facilitating substrate binding. Thus the chaperone function of HSC70 is critically dependent on the interaction with HSP40 (Minami *et al*., 1996).

MGrpE is the bacterial GrpE homolog found in mitochondria (Bolliger *et al*., 1994; Laloraya *et al*., 1994; Nakai *et al*., 1994). MGrpE exists as a dimer and functions as an ADP-ATP exchange factor for MHSP70 (Azem *et al*., 1997). It is also a part of the import complex which helps in the translocation of proteins across the mitochondrial membrane (Kronidou *et al*., 1994; Rassow *et al* .,1994; Schneider *et al*., 1994).

The mouse BAG1 protein (RAP46 being the human analog) has been shown to have a GrpE like function in the eukaryotic cell. It stimulates the release of ADP from HSC70 and thus accelerates the recycling of HSC70 (Höhfeld and Jentsch, 1997).

The eukaryotic cytosol contains two additional proteins which regulate the chaperone activity of HSC70. A two hybrid approach led to the isolation of the 42 kDa `HIP' protein as a HSC70 Interacting Protein in mammals (Höhfeld *et al*., 1995). Functional characterisation of HIP indicated that the regulation of the eukaryotic HSC70 differs from the bacterial HSP70 reaction cycle. HSP40 stimulates the ATPase activity of HSC70 and the ADP bound form with high affinity for the substrate is generated. HIP prevents the dissociation of ADP from HSC70. Thus, by stabilising the ADP bound conformation of HSC70, HIP stabilizes the chaperone substrate complex (Frydmann and Höhfeld, 1997). The ADP-ATP exchange is stimulated by HOP (for Hsp70/ Hsp90 organising protein; called Sti1p in yeast) which was detected to act as an ADP-ATP

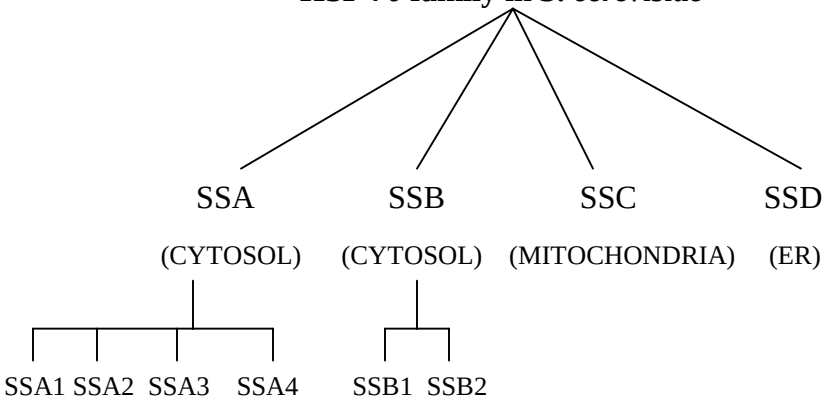
exchange factor in the recycling of HSC70 (Gross and Hessefort, 1996). HOP might serve as a GrpE homolog in the eukaryotic cytosol.

1.3.4 HSP70s in *Saccharomyces cerevisiae*

In the yeast *Saccharomyces cerevisiae* there are four classes of HSP70s: Ssap, Ssbp, Sscp, and Ssdp. Off these four classes the Ssa and Ssb proteins are localised in the cytosol while Ssc is in the mitochondria and Ssd is in the ER.

The cytosolic Ssa proteins are known to assist in the transport of proteins to organelles like the ER, nucleus, peroxisomes and mitochondria. The Ssa subfamily has four members. The Ssa1 and Ssa2 are constitutively expressed while Ssa3 and Ssa4 are stress induced. The two members of the Ssb subfamily Ssb1 and Ssb2 are known to play a role during protein synthesis. The HSP70 family in *S. cerevisiae* has been summarised in the following diagram.

HSP 70 family in *S. cerevisiae*



The *SSA* gene family

MUTANT PHENOTYPE	ESSENTIAL SUBFAMILY
LOCALISATION	CYTOSOL
FUNCTION	HEAT SHOCK REGULATION, TRANSPORT OF PROTEINS,OTHERS?
EXPRESSION AT 23°/ 39°	Ssa1 Ssa2 Ssa3 Ssa4 

Ref: The Biology of Heat Shock Proteins and Molecular Chaperones, Cold Spring Laboratory Press, 1994. Page no. 31-52.

2. AIM OF THE STUDY

Cytosolic HSP70s are known to assist in the transport of proteins into various organelles in the cell like mitochondria, ER, peroxisomes and nucleus. We wanted to determine if the cytosolic HSP70s also assist in the cytosol to vacuole transport of Aminopeptidase 1.

There are two models which have been proposed for the transport of Ape1p to the vacuole:

(1) Seguí-Real *et al*., in 1995 suggested that the transport of Ape1p to the vacuole is by translocation.

(2) It is proposed that Ape1p assembles into a dodecamer in the cytosol and is then transported in a double membrane vesicle (CVT vesicle) to the vacuole.

If the transport of Ape1p is by translocation then the HSP70s could be assisting in the unfolding of the protein before it crosses the membrane. This would be very similar to the role they perform in the transport of proteins into the ER and mitochondria.

However, if the transport of Ape1p is a vesicle mediated process then HSP70s might assist in the assembly of the protein, its packaging into the vesicle, or in the targeting and/or fusion of the vesicle with the vacuole.

Hence irrespective of the mechanism of transport cytosolic HSP70s might be assisting in the transport of Ape1p.

Further, it is known that many of the components required for the cytosol to vacuole targeting pathway are necessary for autophagocytosis also. Hence we wanted to determine if HSP70s are required for both the cvt pathway and autophagocytosis or specifically for one of them.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment used for molecular biology and biochemical work

Analytical weighing balance	
Type 1602 MP and M5P	Sartorius, Göttingen
Autoclave Type Tecnoclav 50	Tecnorama,Zürich, Switzerland
Film developing machine Gevamatic 60	Agfa-Gevaert, Leverkusen
Gel drier	Bio-Rad, Hilden
Heating block 5320	Eppendorf, Hamburg
Ice Machine	Ziegra, Isern
Incubator for bacterial plates	Heraeus
Incubator for bacterial liquid cultures	New Brunswick Sc., Edison/ USA
Magnetic stirrer Ika-Combimag Ret	Janke & Kunkel, Staufen
pH-Meter CG 820	Schott, Göttingen
Power pack	Benedikt Heinemann, Duderstadt
Semi-Dry-Blot system	Benedikt Heinemann, Duderstadt
Sonicator, Sonifer W-450	Brenson Ultrasonic SA, Carouge Geneva/ USA
Spectrophotometer, Uvikon 932	Kontron Instruments
Thermocycler	Eppendorf, Hamburg
Transilluminator IL-400-M	Bachofer, Reutlingen
Vortex Super-Mixer	Lab-line Instruments, Melrose/USA

Weighing Machine Type	
1264 MP and 1265	Sartorius, Göttingen
Centrifuges:	
Biofuge A	Heraeus Sepatech, Osterode/ Harz
Biofuge Fresco	Heraeus Sepatech, Osterode/ Harz
Eppendorf centrifuge 5414 and 5415	Eppendorf, Hamburg
Labofuge	Heraeus Sepatech, Osterode/ Harz
Cooling centrifuge J-21 C	Beckman, München
Ultracentrifuge L8-70M	Beckman, München
Table top Ultracentrifuge TL-100	Beckman, München

Rotors for cooling centrifuges and ultracentrifuge:

JA-10 rotor, upto 17.680x g	Beckman, München
JA-20 rotor, upto 48.300x g	Beckman, München
SW40 rotor, upto 40,000 rpm	Beckman, München
TLA-100.3	Beckman, München

3.1.2 Materials for working with *Saccharomyces cerevisiae*

Culture flasks	Schott, Mainz
Incubator for yeast liquid cultures	Innova 4330, New Brunswick Scientific, Nürtingen
Incubator for yeast plates	Friocell, MMM medcenter.
Microflow Biological Safety Cabinet	Nunc, Wiesbaden.

3.1.3 Materials for molecular biology and biochemical experiments

Glass pipettes	Schütt, Göttingen
Parafilm	American National Can Neenah/ USA
Pasteur pipettes	Schütt, Göttingen
Petridishes for cells	Sarstedt, Braunschweig
Reaction tubes:	
0,2 ml (PCR)	Greiner, Nürtingen
0,5 ml	Sarstedt, Braunschweig
1,5 and 2,2 ml	Greiner, Nürtingen
Sterile filter Ministart NML, 0,45 µM; 0,2 µM	Sartorius, Göttingen
Centrifuge tubes:	
JA-10 Polypropylene tube	SCI Science Service, München
JA-20 Polypropylene tube	SCI Science Service, München
SW 40 Polyallomer	Beckman, München

3.1.4 Materials for Western Blots

Enhancer and Peroxide solutions	Pierce, Illinois/ USA
Nitrocellulose blotting membrane, 0,2µM	Sartorius, Göttingen
Rontgenfilme, XAR-5	Kodak, Stuttgart
Supersignal Chemiluminiscent Substrtate	Pierce, Illinois/ USA
Whatman paper	Schleicher and Schull, Dassel

3.1.5 Enzymes, Standards and Antibiotics

1-kb DNA ladder	Gibco BRL, Eggenstein
Prestained protein molecular weight marker	Calbiochem, Frankfurt
Proteinase K	Boehringer, Mannheim
Restriction endonucleases	New England Biolabs, Bad Schwalbach

3.1.6 Kits for DNA, RNA and Protein

Bio-Rad Protein Assay	Bio-Rad, München
DNA preparation (mini and midi preps)	Qiagen, Hilden
Super Signal CL-HRP Substrate System	Pierce, Rockford/ USA

3.1.7 PCR-Material

Taq-DNA-Polymerase	Pharmacia, Freiburg
dATP	Pharmacia, Freiburg
dCTP	Pharmacia, Freiburg
dGTP	Pharmacia, Freiburg
dTTP	Pharmacia, Freiburg
Taq-Reaction buffer	Pharmacia, Freiburg
Oligonucleotides	NAPS, Göttingen

3.1.8 Antibodies

Primary

Name	**Antigen**	**Immunized Species**
anti-aminopeptidase 1	aminopeptidase 1	Rabbit
anti-hexokinase	hexokinase	Rabbit
anti-carboxypeptidase Y	carboxypeptidase Y	Rabbit
anti-hsp70	Ssa1p and Ssa2p	Rabbit
anti-hsp70	Ssa3p and Ssa4p	Rabbit

All antibodies used were polyclonal.

3.1.9 Secondary antibodies

Goat anti-rabbit Horse Radish Peroxidase tagged	Dianova, Hamburg

3.1.10 Chemicals

Acetic acid	Merck
Acetone	Merck
Acrylamide/ Bisacrylamide (30%/ 0,8%)	Serva
Agar	Sigma
Agarose	Sigma
Ammonium acetate	Merck
Ammonium caproic acid	Sigma
Ammonium chloride	Sigma
Ammonium peroxide sulphate (APS)	Merck

APNE (N-acetyl-phenylalanine-β-naphthyl-ester)	Sigma
Bacto tryptone	Roth
Bacto yeast extract	Roth
Bovine Serum Albumin (BSA)	Serva
Bromophenol blue	Bio Rad
Calcium chloride	Merck
Chloroform	Merck
Coomassie, Serva BlueR	Serva
DEAE dextran	Sigma
Dimethylsulfoxide (DMSO)	Merck
Disodiumhydrogenphosphate	Merck
Dithiothreitol	Serva
Ethanol	Merck
Ethidium bromide	Serva
Ethylenediaminetetraaceticacid -sodium salt (EDTA)	Merck
Fast Garnet GBC salt	Sigma
Ficoll	Sigma
Glucose	Merck
Glycerin	Merck
Glycin	Roth
HEPES (N-2-Hydroxyethylpiperazin-N'-2-ethanesulfonic acid)	Merck
H-Leu pNA	Bachem biochemica, Heidelberg
Hydrochloric acid	Merck
Imidazol	Merck
IPTG	Serva
Isoamylalcohol	Merck
Lyticase	Boehringer

Magnesium chloride	Merck
2-Mercaptoethanol	Sigma
Methanol	Merck
Optiprep (Iodixanol)	Sigma
Peptone	Roth
Phenol	Merck
PIPES (Piperazine-N,N'-bis (2 ethane sulfonic acid)	Sigma
Ponceau stain	Serva
Silver nitrate	Sigma
Sodium acetate	Merck
Sodium azide	Sigma
Sodium chloride	Merck
Sodium dihydrogen phosphate	Merck
Sodium dodedcyl sulphate (SDS)	Sigma
Sodium hydroxide	Merck
Sorbitol	Sigma
Sucrose	Merck
Sulphuric acid	Merck
TEMED (N'N'N'N Tetramethylethylenediamine)	Sigma
Trichloroacetic acid	Merck
Tris- base	ICN Biomedicals, Aurora/ USA
Triton X-100	Sigma
Tween-20	Sigma
Yeast extract	Roth
Yeast nitrogen base without amino acids	Gibco BRL
Zymolyase 20T	Seikagaku corporation

All chemicals were of the pro analysis grade and obtained from: Boehringer, Mannheim; Gibco BRL, Eggenstein; Merck, Darmstadt; Serva, Heidelberg; Pharmacia, Freiburg; BioRad, München; Seikagaku corporation, Tokyo, Japan; and Sigma, München.

3.1.11 Amino acids

All amino acids were purchased from Sigma.

3.1.12 Protease inhibitors

Antipain	Sigma
Chymostatin	Sigma
Elastin	Sigma
Leupeptin	Sigma
Pepstatin	Sigma

3.1.12 Yeast Strains used

Wild type	Mata his3 leu2 lys2 Δtrp1 ura3
Δssa1	Matα his3-11, 3-15 leu2-3,2-112 ura3-52 trp1-Δlys2 ssa1-3 ssa1::HIS3
Δssa2	Matα his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa2-2 ssa2::URA
ΔssaI/II	Matα his3 leu2 lys2 Δtrp1 ura3 ssa1::HIS3 ssa2::LEU2
ydj1	Matα ade2-1 leu2-3.112 his3-11.15 trp1-1 ura3-1 can1-100 ydj1-2::HIS3 LEU2::ydj1-151

3.1.13 Bacterial strains used

M15 (pREP4)	K12(Nal^{s}, Str^{s}, Rif^{s}, Lac^{-}, Ara^{-}, Gal^{-}, Mtl^{-}, F^{-}, RecA^{+}, Uvr^{+}, Lon^{+})
DH5α	F'/*endA1, hsdR17*($r^{-}_{k}m^{+}_{k}$), *supE44, thi-1, recA1,gyrA*(Nal^{r})*relA1Δ(lacZY-argF*),U169*deoR*(Φ80*dlacΔ(lacZ*)M15)

3.1.14 Media for growing *Saccharomyces cerevisiae* cells

1. YPD Media	20 g	Peptone
	10 g	Yeast extract
	20 g	Dextrose

Volume made up to 1000 ml with water

2. YPUAD media	2 %	Yeast extract
	2 %	Peptone
	40 µg/ ml	Uracil
	40 µg/ ml	Adenine
	2 %	Glucose

3. Synthetic minimal media (SD)	6.7 g	Yeast nitrogen base (without amino acids)
	20 g	Dextrose

Amino acid concentration according to Guthrie and Fink., Methods in Enzymology, 1991.

Volume made up to 1000 ml with water.

4. Synthetic minimal media deficient in nitrogen (SD-N)

0.17 g	Yeast nitrogen base (without amino acids)

The remaining preparation was as described above for synthetic minimal media.

Amino acid	**Final Concentration mg/ litre**	**Stock per 100 ml**
Adenine sulphate and Uracil	20	200 mg
L-Tryptophan, L-Histidine-HCl, L-Arginine-HCl and L-Methionine	20	1 g
L-Tyrosine	30	200 mg
L-Leucine, L-Isoleucine and L-Lysine HCl.	30	1 g
L-Phenylalanine	50	1 g
L-Glutamic acid and L-Aspartic acid	100	1 g
L-Valine	150	3 g
L-Threonine	200	4 g
L-Serine	400	8 g

3.1.15 Media for growing Bacterial cells

Luria Bertani (LB) Media	10 g	Bacto tryptone
	5 g	Bacto yeast extract
	10 g	Sodium chloride

pH set to 7.5 with Sodium hydroxide. Volume made up to 1000 ml with water

Antibiotics

1. Ampicillin 25 mg/ ml

Dissolved in water. pH set to 8.0 with 10 N Sodium hydroxide. Filter sterilized and aliquots stored at -20°C.

Working concentration 100 µg/ ml

2. Kanamycin 25 mg/ml

Dissolved in water. Sterile filtered and stored aliquots at -20°C.

Working concentration 50 µg/ ml

3.1.16 Stock solutions and buffers

1 M DTT	3,08 g	Dissolved in 20 ml of 0,01 M sodium acetate pH 5,2. Filter sterilized and aliquots stored at -20°C.
0,5 M EDTA	181,1 g	Ethylene Diamine Tetra Acetic acid (sodium salt) 2 x H_2O dissolved in

		800 ml of water. pH set to 8,0 with NaOH. Volume made up to 1000 ml and autoclaved.
Ethidium Bromide	0,2 g	Dissolved in 20 ml of water. Stored at 4°C.
0,1 M IPTG	23,8 mg	Dissolved in 1 ml of water. Sterile filtered and aliquots stored at -20°C.
10 x PBS	80 g	Sodium chloride
(Phosphate Buffer Saline)	1,6 g	Disodiumhydrogen phosphate dissolved in 800 ml water. volume was made up to 1000 ml.
100 mM K-PIPES	37,85 g	Dissolved in 80 ml of water. pH set to 6,8 and volume made up to 100 ml. Filter sterilized.
Protease Inhibitor mix	1,25 mg/ml	Leupeptin
	0,75 mg/ml	Antipain
	0,25 mg/ml	Chymostatin
	0,25 mg/ml	Elastin
	5,0 mg/ml	Pepstatin dissolved in DMSO. Aliquots stored at –20°C.

RNase free of DNase	10	mg/ ml	Pancreatic RNase (RNase A) was dissolved in 10 mM Tris-Cl pH 7,5 and 15 mM NaCl. The solution was heated at 100°C for 15 min and allowed to cool to room temperature. Aliquots were stored at -20°C.
3M Sodium acetate (M.W. 82,03)			Dissolved in water and adjusted the pH to 5,2 with glacial acetic acid. Sterilized by autoclaving.
2,5 M Sorbitol	227.7	g	Dissolved in water. Volume made up to 500 ml and sterilized by autoclaving.
50 x TAE	2	M	Tris-Base
	0,1	M	EDTA
			set pH to 8,0.
TBS (1x)	150	mM	Sodium chloride
(Tris Buffer Saline)	10	mM	Tris/ HCl pH 7,4
TE	10	mM	Tris/ HCl pH 7,5
	1	mM	EDTA
1 M Tris/ HCl	121,1	g	Tris-Base dissolved in 800 ml water. pH was set to the required value with conc.

		HCl. Volume was made up to 1000 ml and autoclaved.
1 M Tris/ SO_4	121.1 g	Tris-base dissolved in 800 ml of water. pH was set to 9.4 using conc. H_2SO_4. Volume was made up to 1000 ml.

3.1.17 Computing facilities used

Hardware	Apple Macintosh 7600/ 132	Apple Macintosh
	Apple Laser Writer 16/ 600 PS	Apple Macintosh
	IBM compatible	
	Satellite 2060 CDS	Toshiba
	Scan Jet 4c/T	Hewlett Packard
Software	Adobe Photoshop 4.0	Adobe
	Word 6.0	Microsoft
	Canvas 5.0	Deneba Canvas
	Word for Windows	Microsoft Corporation

3.2 Molecular Biology Methods

3.2.1 Isolation and analysis of DNA

3.2.1.1 Mini preparation of plasmid DNA

(Qiagen-handbook, April 1997)

P1:	50	mM	Tris/ HCl pH 8,0
	10	mM	EDTA
	100	µg/ ml	RNase A
P2:	0,2	M	NaOH
	1	%	SDS
P3:	3	M	Potassium acetate pH 5,5

A single *E. coli* colony was inoculated into 5 ml of LB media containing 100 µg/ ml of ampicillin. The culture was grown overnight at 37°C in a shaking incubator. Cells were pelleted in an Eppendorf centrifuge at 1800xg (6000 rpm) for 5 min. Cell pellet was resuspended in 250 µl of buffer P1. To this 250 µl of buffer P2 was added and mixed by gently inverting the tube 4-6 times. 350 µl of buffer P3 was added and gently mixed. This was centrifuged for 10 min at 12000 rpm. The supernatant was applied onto a QIAprep spin column and centrifuged for 1 min at 12000 rpm. The flow through was discarded. The column was washed with 0,75 ml of buffer PE and centrifuged again for 1 min. The flow through was discarded. The column was placed in a clean eppendorf tube and 50 µl of double distilled water was added. The column was let to stand for 1 min and the DNA was eluted by centrifuging at 12000 rpm for 1 min.

3.2.1.2 Midi preparation of plasmid DNA

(Qiagen-handbook, April 1997)

Composition of buffer P1, P2 and P3 used in this preparation have been described in section 3.2.1.1.

QBT:	750	mM	Sodium chloride
	50	mM	MOPS pH 7,0
	15	%	Ethanol
	0,5	%	Triton X-100
QC:	1	mM	Sodium chloride
	50	mM	MOPS pH 7,0
	15	%	Ethanol
QF:	1,25	M	Sodium chloride
	50	mM	Tris/ HCl pH 8,5
	15	%	Ethanol

E. coli was inoculated into 100 ml LB media containing 100 μg/ ml ampicillin. The culture was grown at 37°C overnight in a shaker incubator. Cells were pelleted in a Beckman JA10 rotor at 8000 rpm for 5 min. Pellet was resuspended in 4 ml of buffer P1. 4 ml of buffer P2 was added and gently mixed. This was incubated at room temperature for 5 min. 4 ml of buffer P3 was added, mixed gently, and incubated on ice for 15 min. Cell lysate was centrifuged at 13000 rpm for 30 min at 4°C in the Beckman JA20 rotor. Qiagen tip 100 was equilibriated using 4 ml of buffer QBT. The flow through was discarded. The supernatant from the centrifugation was applied onto the column. The column was washed with 10 ml buffer QC, two times. DNA was eluted using 5 ml of buffer QF. 3,5 ml (0,7 volumes of the eluate) of isopropanol was added to precipitate the DNA. DNA was pelleted by

centrifuging at 13000 rpm for 30 min at 4°C in the Beckman JA20 rotor. The pellet was resuspended in 0,5 ml of water. To this 166 µl of 7M ammonium acetate and 1 ml of 100 % ethanol was added and incubated at -80°C for 30 min. DNA was pelleted at 14000 rpm for 10 min at 4°C. DNA was then washed with 0,5 ml 70 % ethanol and pelleted at 14000 rpm for 10 min at 4°C. DNA pellet was dried and dissolved in 50 µl of double distilled water.

3.2.1.3 Precipitation of DNA with ethanol

Two volumes of 100 % ethanol was added to the DNA . This was incubated at -70°C for 20 min or 10 min on dry ice or overnight at -20°C. DNA was pelleted at 13000 rpm for 10 min. The pellet was washed with 70 % ethanol and pelleted by centrifuging for 5 min at 13000 rpm. The DNA pellet was dried under vacuum for 3 min.

3.2.1.4 DNA extraction using Phenol/ chloroform/ isoamylalcohol

Phenol/ chloroform/ isoamylalcohol (PCI) in the ratio 25:24:1 is used to remove proteins from the DNA preparation. Cell lysate was treated with an equal volume of the PCI solution. The contents were mixed and centrifuged at 13000 rpm for 5 min. The aqueous phase was further used for precipitating DNA.

3.2.1.5 Determining the concentration of DNA

DNA concentration was determined using a spectrophotometer at 260 nm. DNA was diluted in water and the concentration measured at 260 nm. An

optical density (O.D) of 1 at 260 nm corresponds to 50 µg/ ml of double stranded DNA.

3.2.1.6 Restriction Digestion of DNA

The activity of restriction enzymes is measured in terms of `Units' (U). One unit of restriction enzyme digests 1µg of substrate DNA in 1 hour.

Plasmid DNA	0,5-1 µg
10X buffer	1 x
Restriction enzyme	1-2 U
BSA	1 µg/ µl
(added where essential)	
Water	Added to obtain a final volume of 20 µl.

Reaction mix was incubated at 37°C for 2 hours. Incubation temperature was changed depending on the enzyme used (referred New England Bio Labs Beverly U.S.A. catalogue). An aliquot of the reaction mix was analysed on an agarose gel.

The buffers and enzymes used were all from New England Biolabs. The composition of the buffers is as follows

NEB 1: 50 mM Tris/ HCl (pH 7) + 10 mM $MgCl_2$ + 1 mM DTT
NEB 2: 50 mM Tris/ HCl (pH 8) + 10 mM $MgCl_2$ + 1 mM DTT + 50 mM NaCl
NEB 3: 50 mM Tris/ HCl (pH 8) + 10 mM $MgCl_2$ + 1 mM DTT + 100 mM NaCl
NEB 4: 50 mM Tris/ HCl (pH 8) + 10 mM $MgCl_2$ + 1 mM DTT + 50 mM KAc

3.2.1.7 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is used to analyse the size and purity of DNA. Depending on the size of the DNA fragment the concentration of agarose used is varied.

Agarose concentration (%)	DNA size (kb)
0,6	20 - 1
0,9	7 - 0,5
1,2	6 - 0,4
1,5	4 - 0,2
2,0	3 - 0,1

Gel loading buffer	0,05 % (w/v)	Bromophenol blue
	0,05 % (w/v)	Xylenecyanol
	15 % (w/v)	Ficoll

The required quantity of agarose was weighed, to this 60 ml of 1x TAE was added and boiled. The agarose solution was allowed to cool till about 55°C and ethidiumbromide was added to a final concentration of 0,5 μg/ ml. This was poured into the agarose gel chamber and allowed to polymerise completely. To the DNA probe 10-20 % of the gel loading buffer was added. The sample was loaded into the lane using a pipette. The gel electrophoresis was carried out at 3-4 V/ cm. Ethidium bromide added to the gel intercalates in the DNA and hence DNA could be analysed under a UV-transilluminator. The gel was documented using the video system connected to the UV transilluminator.

3.2.2 Transformation of cells

3.2.2.1 Transformation of bacterial cells

3.2.2.1.1 Chemical transformation of bacterial cells

Medium A	10 mM	Magnesium sulphate x 7 H_2O
	0,2 %	Glucose
	Volume made up with Luria Bertani media and sterile filtered.	
Solution B	36 %	Glycerol
	12 %	PEG (MW 7500)
	12 mM	Magnesium sulphate x 7 H_2O
	Volume made up with LB media and pH was set to 7,0 with NaOH. Sterile filtered.	

A 2-3 ml bacterial pre culture was grown in LB media at 37°C overnight. 1 ml of the preculture was then inoculated into 99 ml of Medium A and grown at 37°C till an $O.D_{600}$ of 0,4 - 0,6. Cells were pelleted at 4000 rpm for 5 min at 4°C and resuspended in 1 ml of ice cold medium A. To this 2,5 ml of solution B was added and mixed carefully. To 100 µl of the cells 50-75 ng of DNA was added. Cells were incubated on ice for 20 min and then heat shocked at 42°C for 2 min. Cells were then again incubated on ice for 20 min. 0,2 ml of LB media was added and the cells were allowed to revive at 37°C on the shaker for 1 hour. Cells were plated on LB plates containing the required antibiotic.

3.2.2.1.2 Electroporation of bacterial cells

10 % Glycerol	1.26 g of glycerol in 10 ml water.	
SOC medium	0,5 %	Yeast extract
	2 %	Bacto-tryptone
	10 mM	Sodium chloride
	2,5 mM	Potassium chloride
	10 mM	$MgSO_4$ x H_2O
	10 mM	Magnesium chloride
	20 mM	Glucose

1. Preparation of electrocompetent cells:

A single *E. coli* colony was inoculated into 5 ml of LB media and allowed to grow overnight at 37°C in a shaker incubator. 2,5 ml of this pre culture was inoculated into 500 ml LB media and allowed to grow to an $O.D._{600}$ of 0,5-0,7 at 37°C. Cells were prechilled on ice for 15 min and then pelleted at 5000 rpm for 15 min at 4°C. Pellet was resuspended in 500 ml of ice cold water and centrifuged as described above. The washing was repeated one more time. To the pellet an equal volume of water was added and resuspended (generally a 500 ml culture yielded a pellet of volume ~500 µl).

2. Transformation of the electrocompetent cells

For each electroporation 200 µl of the electrocompetent cells were used. To these cells 0,5 µg DNA was added and the contents were transferred into an electroporation cuvette. The cuvette was chilled on ice before the cells and DNA were added. The cuvette was then placed in the electroporater and the pulse was applied. SOC media was added to the cells and the cells were allowed to grow in sterile tubes for 1 hour at 37°C in a shaker incubator. Cells were then plated on LB plates containing the required antibiotic.

3.2.2.2 Transformation of yeast cells

3.2.2.2.1 Chemical transformation of yeast cells

10 x TE buffer	0,1 M Tris/ Cl 0,01 M EDTA, pH 7,5
10 x LiOAc	1 M Lithium acetate, pH 7,5 (pH adjusted using diluted acetic acid)
Salmon sperm DNA .	10 mg/ ml Dissolved in water. Sonicated and denatured for 5 min at 95°C. Chilled on ice.

Cells were grown in YPD media to an O.D $_{600}$ 0,6-1,2 and pelleted at 3000 rpm for 5 min at 4°C. Cells were washed four times in sterile water.
Cell pellet from a 200 ml culture was resuspended in 1,5 ml LiOAc/ TE (1x) and incubated on ice for 2 hours. To 100 µl of the competent cells 1-5 µg of plasmid DNA and 100 µg of salmon sperm DNA was added and incubated at 30°C for 30 min. 1,2 ml of 40 % PEG 4000 in 1x LiOAc/ TE was added to each of the tubes and carefully mixed by pipetting. Cells were incubated at 30°C for 30 min with intermittent mixing. Cells were heat shocked at 42°C for 5 min and then allowed to cool on ice. Cells were pelleted by very brief centrifugation. The cell pellet was resuspended in 1 ml of 1x TE buffer. 0,2 ml of cells were spread over the selection plate.

3.2.2.2.2 Electroporation of yeast cells

1. Preparation of electrocompetent cells

Yeast cells were grown in YPD media to an O.D. $_{600}$ of 1,3-1,5 at 30°C in a shaker incubator. Cells were pelleted at 4000xg for 5 min at 4°C. Cells were washed with 500 ml cold sterile water. Cells were pelleted as described before and washed again with 250 ml ice cold sterile water. Cell pellet was then resuspended in 20 ml ice cold sorbitol and centrifuged as decribed above. The cell pellet was resuspended in 0,5 ml 1 M sorbitol.

2. Transformation of electrocompetent yeast cells:

To 65 µl of electrocompetent cells 0,1 µg of DNA was added and transferred to the prechilled electroporation cuvette. The cuvette was incubated on ice for 5 min and then placed in the electroporator. A pulse of 1500 V was applied. The cuvette was then removed and 1 ml of 1 M sorbitol was added to it. Cells were then plated on selective media plates containing 1 M sorbitol.

3.2.3 Entering new strains into the stock collection

3.2.3.1 Glycerol stocks of bacterial strains.

Bacterial cultures were grown overnight at 37° C in a shaker incubator. 0,9 ml of sterile 50 % glycerin was taken into each of the vials. To this 2,1 ml of the overnight culture was added. The contents were gently mixed and placed on dry ice for a few minutes. The vials were then stored at -80° C.

3.2.3.2 Glycerol stocks of yeast cells

Yeast cells were spread onto a YPD plate and the cells were allowed to grow at 30°C. 3 ml of sterile 15 % glycerol was taken in a vial. Half of the lawn of cells on the plate was scraped of using a small sterile spatula and resuspended into the glycerol. The vials were then placed directly at -70° C and the cultures were allowed to freeze slowly.

3.3 Biochemical methods

3.3.1 Spheroplasting yeast cells

Tris SO_4 DTT buffer:	0,1	M	Tris SO_4 pH 9.4
	10	mM	DTT
Spheroplasting buffer:	1,2	M	Sorbitol
	50	mM	Tris/ Cl pH 7.5
	10	mM	EDTA

Yeast cells are surrounded by a firm cell wall. The cell wall has to be removed before performing biochemical experiments with the cells. After the cell wall is removed the cells are called spheroplasts and the process is called spheroplasting.

The cell pellet was resuspended in Tris SO_4 DTT buffer at a final concentration of 10 $O.D._{600}$/ ml and incubated at 30°C for 15 min with slow shaking. Cells were pelleted at 3000 rpm for 5 min. The pellet was resuspended in spheroplasting buffer at a concentration of 6 $O.D._{600}$/ ml. An aliqout of the resuspended cells was retained on ice to determine the spheroplasting (NS) efficiency. 0,5 mg/ 50 $O.D_{600}$ of Zymolyase 20 T was added and the cells were

incubated at 30°C for 30 min with slow shaking. An aliqout of the cells after spheroplasting was also taken (S). The two samples were diluted with water and the optical density was measured at 600 nm. The O.D. value obtained for the spheroplasted sample (S) indicates the amount of non spheroplasted cells. Using this the percentage of spheroplasting or the spheroplasting efficiency can be determined.

Calculation of spheroplasting effeciency:

O.D. of cells in spheroplasting buffer (NS)
- O.D of cells after spheroplasting step (S)
Cells which are spheroplasted .

Percentage of spheroplasting obtained/ Spheroplasting effeciency

$$= \frac{\text{Cells which are spheroplasted}}{\text{O.D. of cells in spheroplasting buffer (NS)}} \times 100$$

3.3.2 Preparation of yeast cell lysates

3.3.2.1 Preparation of cell lysates using glass beads

Glass beads: Glass beads were immersed in conc. HCl overnight. The beads were then washed in distilled water till the pH reached 7. These beads were then autoclaved and stored at 4°C.

Yeast cells were grown at 30°C in a shaker incubator in either minimal media or YPD. Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted and pelleted at 2000 rpm for 6 min. Spheroplasts were

resuspended in 700 µl of 0.1 M sodium phosphate buffer. To this protease inhibitor mix was added to a final concentration of 1x. The spheroplasts were incubated in the cold room on a rotating wheel for 10 min. Spheroplasts were lysed using acid washed and cooled glass beads by vortexing at maximum speed for 6 min with intermittent cooling on ice. The unlysed cells and glass beads were pelleted by centrifuging at maximum speed for 3 min. The supernatant, cell lysate, was collected and the protein content was estimated. Proteins were precipitated and further analysed.

3.3.2.2. Preparation of yeast cell lysates using DEAE dextran

Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted as described above. Spheroplasts were pelleted at 2000 rpm for 2 min. Spheroplasts were resuspended in 200 mM sorbitol and 10 mM PIPES, pH 6,8. DEAE dextran was added to a final concentration of 50 µg/ 100 O.D $_{600}$ (concentration of DEAE dextran had to be standardized for each strain). Spheroplasts were incubated on ice for 1 min and then at 30°C for 5 min.

3.3.2.3 Lysing spheroplasts using water

Cells were spheroplasted as described in 3.3.1. Water was added to the spheroplasts in the ratio 1:3. The contents were gently mixed and resuspended. The suspension was incubated on ice for 5 min. This method of cell lysis could however lead to the lysis of some internal membranes as well.

3.3.3 Precipitation and solubilisation of proteins

3.3.3.1 Precipitation of proteins using methanol

To one volume of the cell lysate twice the volume of methanol and two thirds the volume of chloroform and water was added. The contents were vortexed and centrifuged at 13000 rpm for 2 min. The protein pellet was washed with 1 ml methanol.

3.3.3.2 Precipitation of proteins using Tri Chloro Acetic acid (TCA)

To the sample 50 % TCA was added to a final concentration of 10 %. Samples were incubated on ice for 30 min and centrifuged at 13000 rpm for 30 min at 4°C. The protein pellet was washed with 1 ml cold acetone.

3.3.3.3 Solubilisation of proteins

3x Laemmli buffer	100	mM	Tris/ Cl 6,8
	6	%	SDS
	0.3	%	Bromophenol blue
	30	%	Glycerol
	3	%	β mercaptoethanol

Proteins were resuspended in 1x Laemmli buffer. Proteins were resuspended by sonicating. The resuspended proteins were boiled at 95°C for 10 min.

3.3.4 Estimation of protein content using the BIORAD reagent

Bovine Serum Albumin (BSA) stock solution 1 mg/ ml

Range 1-20 µg/ µl

Standard curve was made using BSA in the range of 1-20 µg. 10 µl of each of the samples was used for the estimation. Volume was made upto 800 µl using water. 200 µl of the BIORAD reagent was added and the samples were incubated for 5 min at room temperature. The optical density was measured at 595 nm.

3.3.5 Analysis of proteins

3.3.5.1 SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE) for separation of proteins

Gel running buffers

Anode buffer	50 mM	Tris
	384 mM	Glycin
		Volume was made up in double distilled water.
Cathode buffer	50 mM	Tris
	384 mM	Glycin
	0,1 % (w/v)	SDS
	0,001 % (w/v)	Bromophenolblue
		Volume was made up in double distilled water.

Separating gel buffer	1,5 M	Tris/ Cl pH 8.8
	0,4 % (w/v)	SDS
		Volume was made up in double distilled water.
Stacking gel buffer	0,5 M	Tris/ Cl pH 6.8
	0,4 % (w/v)	SDS
Acrylamide Solution	30 % (w/v)	Acrylamide
	0,8 % (w/v)	Bisacrylamide
		Volume made up in double distilled water.
Ammonium per sulphate	10 % (w/v)	recrystallised ammonium persulphate (APS) in double distilled water.
4% stacking gel	1,3 ml	Acrylamide
	2,5 ml	Stacking gel buffer
	100 µl	APS
	10 µl	TEMED
	6,1 ml	Double distilled water.

Separating Gel

Poly acrylamide conc.	**7,5%**	**9%**	**10%**
Acrylamide solution (ml)	7,9	9,5	10,5
Separating buffer (ml)		7,5	
APS (µl)		250	
TEMED (µl)		25	
Water		Final volume 30 ml	

SDS PAGE is a biochemical technique where in proteins are separated based on their molecular weight. The gel is discontinuous in that it has a stacking gel which is present on top of the separating gel. SDS PAGE can be used to separate proteins based on their native size (native SDS PAGE) or their sub unit size (reducing SDS PAGE).

The two glass plates were cleaned using ethanol and fixed together using clips. The edges were sealed using agarose. This was done to ensure that the solutions do not leak. The separating gel was poured first and isopropanol was layered on top of it. The gel was allowed to polymerise for about 30 min. Then the isopropanol was thoroughly washed off using double distilled water. Stacking gel was then poured on top of the separating gel and a comb of the appropriate size and wells was placed in this gel. The gel was allowed to polymerise for about 30 min. After polymerisation combs were removed and the wells were cleaned with double distilled water to ensure that there was no acrylamide within the wells. The gel was placed in the gel running chamber. Anode buffer was poured into the bottom well of the chamber and cathode buffer into the upper well of the chamber. Protein samples boiled in the solubilisation buffer were loaded into the wells. The gel was connected to a power supply and allowed to separate at 60 mA/ h at room temperature.

3.3.5.2 Coomassie blue staining of Polyacrylamide gels

Coomassie blue stain	0,5 % (w/v)	Coomassie blue
	50 % (v/v)	Ethanol
	10 % (v/v)	Acetic acid
		in double distilled water
Destaining solution	50 %	Methanol
	10 %	Acetic acid
		in double distilled water.

The gel was stained in Coomassie blue solution at room temperature for 1-2 hours. The gel was then destained using the destaining solution overnight. After complete destaining the gel was washed with double distilled water for 15 min and then dried using a gel drier (BioRad).

3.3.5.3. Western blot analysis (semi dry method)

Semidry blot buffers

Cathode buffer	40 mM	Ammonium caproic acid
	20 mM	Tris/Cl
	20 %	Methanol
		Dissolved in water and pH set to 9,0. The volume was made up with water.
Anode buffer	75 mM	Tris/Cl
	20 %	Methanol

	Dissolved in water. pH set to 7,4 and volume made up with water.
Tris Buffer Saline Tween (TBST)	TBS containing 0,1 % Tween 20.

Primary antibodies used	**Final concentration used**
anti-aminopeptidase 1	1:2000
anti-carboxypeptidase Y	1:5000
anti-hexokinase	1:1000
anti-Ssa1p and Ssa2p	1:10000
anti-Ssa3p and Ssa4p	1:6000

Secondary antibody used	**Final concentration used**
HRP (Horse Raddish Peroxidase) tagged anti-rabbit goat antibody	1:10000

Proteins were separated by SDS PAGE. Whatman paper was cut 13 cm/ 9 cm. Three sheets were immersed in the semidry anode buffer and three in the semidry cathode buffer. The blot chamber was cleaned using water before starting the set up. Three sheets dipped in the anode buffer were first placed in the chamber. A glass pipette was rolled on it to remove any air bubbles which could be trapped. Then the nitrocellulose membrane dipped in the cathode buffer was placed on top of it and again the air bubbles were removed. The gel was also soaked in the cathode buffer before placing on the nitrocellulose membrane. Next the three sheets dipped in cathode buffer were placed on top. The glass pipette was again rolled on the set up to remove air bubbles. The

proteins were transferred onto the nitrocellulose membrane from cathode to anode using current at 1 mA/ sq.cm. for 1 hour and 10 min.

After transfer was completed the membrane was incubated in TBST containing 5 % milk powder for 1 hour at room temperature or over night in the cold room. After blocking the membrane primary antibody was added, in TBST/ milk powder, at the concentrations mentioned above. The membrane was incubated in the primary antibody for 1 hour. The non specifically bound antibody was washed off using milk powder containing buffer. These washings were done 3 times 10 min each. The membrane was then incubated in the secondary antibody for 1 hour at the concentration mentioned above. Membrane was again washed with the milk powder buffer 4 times, 10 min each and then 2 times with TBST without milk powder. Then the signals were detected.

3.3.5.4.Detection techniques used

1. Chemiluminescent Substrate:

Enhancer and peroxide solutions were used in a 1:1 ratio and incubated at room temperature for 10 min before detecting signals on an X-ray film.

2. Luminol Detection system:

Solution 1 (10 ml)	100 µl	250 mM Luminol
	44 µl	90 mM p-coumaric acid
	1 ml	1 M Tris/Cl pH 8,5
	8,85 ml	Water
Solution 2 (10 ml)	6 µl	30 % Hydrogen peroxide
	1 ml	1 M Tris/Cl pH 8,5

9 ml	Water
Luminol (3 aminophthalhydrazide)	0,44 g was dissolved in 10 ml of DMSO and aliquots were stored at -20°C.
p-coumaric acid	0,15 g dissolved in 10 ml DMSO. Aliquots stored at -20°C

Solution 1 and solution 2 were mixed just before incubating the blot. Solutions were added onto the blot and incubated at room temperature for 1 min. The solution was completely drained off to prevent a high background and then wrapped in polythene. X-ray films were then used for different time periods to detect the signals.

3.3.5.5 Stripping the antibody from the membrane

The membrane was washed in TBST for 10-15 min. Then the membrane was washed with water for 5 min. Membrane was stripped using 0,2 N NaOH for 10 min on a shaker. The membrane was again washed for 5 min with water. The membrane was blocked using TBST containing 5 % milk powder and the antibody incubations were performed as described above.

3.3.6 Glycerol Density Gradients

(reference: Kim *et al* 1997)

Glycerol solutions

20 %	2.52 g glycerol in 10 ml 20 mM PIPES, pH 6,8
30 %	3.78 g glycerol in 10 ml 20 mM PIPES, pH 6.8

40 %	5.04 g glycerol in 10 ml 20 mM PIPES, pH 6,8
50 %	6.30 g glycerol in 10 ml 20 mM PIPES, pH 6.8

Cells were grown to a mid log phase at 30°C in a shaker incubator. Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted using the protocol described in 3.3.1. Spheroplasts were then lysed using glass beads as described in 3.3.2.1. The protein content of the cell lysate was determined using the BIORAD method (3.3.4). 20-50 % glycerol gradients were made using 450 µl of each of the solutions. The gradients were allowed to linearise for 4 hours in the cold room. 1200 µg of protein was loaded onto the gradient. The gradients were centrifuged at 55,000 rpm (260000xg) for 4 hours at 15°C using the Beckman TLS55 rotor. 10 fractions were collected from each of the gradients and TCA precipitated. The proteins were solubilised in Laemmli buffer and analysed by SDS PAGE followed by western blot analysis.

3.3.7 Cell Fractionation

3.3.7.1 Fractionation of cells using ficoll gradients

(reference: Haas, A, 1995).

Ficoll 400 is a neutral, highly branched hydrophilic polymer of sucrose which is used for the isolation of organelles and cells. Density ranges up to 1,2 g/ ml can be obtained. Lower osmotic pressure than sucrose solutions of equal density result in better preservation of functional and morphological integrity of isolated organelles.

Discontinuous gradient centrifugation was used for cell fractionation. The abrupt changes in Ficoll 400 density allow the organelles to be present as sharp bands at the interface between layers of different density.

Ficoll gradient solutions:

12%, 8%, 4% ficoll solutions were made using 200 mM sorbitol and 10 mM PIPES pH 6,8.

Cells were grown to mid log phase at 30°C in a shaker incubator. Cells were spheroplasted as described in 3.3.1. Spheroplasts were pelleted at 2000 rpm for 2 min at 4°C in the Beckman JA20 rotor. Spheroplasts were lysed with water (3.3.2.3). To this 40% ficoll, 2,5 M sorbitol and 100 mM PIPES, pH 6,8 were added to obtain a final concentration of 12 %, 200 mM and 10 mM respectively. Cell lysate was loaded onto a SW40 rotor tube. This was overlaid with 2 ml of 8 % ficoll, 2 ml of 4 % Ficoll and 1 ml of 0 % ficoll. The gradients were centrifuged at 30000 rpm (160000xg) for 90 min at 4°C. 0 %, 0-4 % interface, 4-8 % interface, 8-12 % interface and pellet were collected and TCA precipitated. Proteins were analysed by SDS PAGE followed by western blot analysis.

3.3.7.2 Separation of vacuolar vesicles and subvacuolar vesicles

Cells were grown to a mid log phase at 30°C and vacuoles were isolated as described in 3.3.7.1. Vacuoles in the 0-4 % interface were carefully collected using a syringe. 300 µl of 37 % optiprep in 10 mM PIPES, pH 6,8 and 1 ml of 19 % optiprep in 10 mM PIPES, pH 6,8 were loaded into a TLS 55 rotor tube. This was overlaid with the vacuoles collected from the Ficoll gradient. The samples were centrifuged for 1 hour at 43000 rpm (159000xg) at 12°C in the Beckman table top ultracentrifuge. The different fractions were collected and TCA precipitated. Proteins were separated by SDS PAGE followed by western blot analysis.

3.3.8. Cell fractionation using OPTIPREP (Iodixanol) gradients

Optiprep is a solution of Iodixanol in water. Iodixanol is the trivial name of 5,5'-[(2-hydroxy-1-3-propanediyl)-bis(acetylamino)]bis[N,N'bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenecarboxamide]. Solutions of Iodixanol have a density and refractive index very similar to another gradient material Nycodenz. However Iodixanol has the advantage in that it has an osmolality half that found in Nycodenz. This is very important for the isolation of organelles.

Optiprep can be obtained as a sterile 60 % (w/v) solution of Iodixanol in water. This has a density of 1.320 g/ml.

Optiprep solutions:

37 %, 30%, 25 %, 19 % optiprep solutions were made in 10 mM PIPES, pH 6,8.

OPTIPREP sedimentation gradients

Yeast cells were grown to an $O.D._{600}$ of 0.8 at 30°C in a shaker incubator. Cells were pelleted at 5000 rpm for 5 min in the Beckman JA10 rotor. Cells were spheroplasted using Zymolyase 20T as described in 3.3.1. Spheroplasts were lysed using water. Optiprep gradient containing 3 ml of 37 % Optiprep, 2 ml each of 30 %, 25 % and 19 % was made and allowed to linearise for 1 hour in the cold room. The cell lysate was then loaded on top of the gradient. The gradient was centrifuged at 25000 rpm (111000xg) for 4 hours at 4°C in the Beckman SW40 rotor. The fractions were collected and TCA precipitated. Proteins were analysed by SDS PAGE followed by western blot analysis.

3.3.9 Protease protection experiments

Cells were grown to an $O.D_{600}$ of 0,8. Cells were spheroplasted as described previously (3.3.1). The cell membrane of the spheroplasts were lysed using DEAE dextran (3.3.2.2). An aliquot was directly precipitated using 20 % TCA and 80 % acetone mix. The internal membranes of the remaining aliquots were disrupted using either of the following methods

(i) Using detergent- 0.2 % Triton X-100

(ii) By freezing the cell lysate in liquid nitrogen and then thawing them at 37°C. These freeze thaw cycles were repeated 3 times.

(iii) By freezing the cell lysate in liquid nitrogen and then thawing them by sonication. The freeze thaw sonication was repeated three times.

These samples were then treated with 50 μg/ ml of proteinase K. One sample was treated with 50 μg/ ml of proteinase K, while the internal membranes were kept intact. The proteinase K treated samples were incubated on ice for 30 mins. The proteinase K activity was stopped by adding PMSF to a final concentration of 1 mM and incubating the samples on ice for a further 5 min. The proteins were precipitated using an equal volume of cold 20 % TCA and 80 % acetone mix. The contents were thoroughly mixed by vortexing. Samples were incubated on ice for 30 min and then pelleted at maximum speed for 30 min at 4°C. Protein pellet was washed with cold acetone and then solubilised in 1x Laemmli buffer as described above.

3.3.10 APNE assay

APNE- N-acetyl-phenylalanine-β-naphthyl-ester

(Ref: Wolf and Fink, 1975).

Cells were grown on YPD plates over night at 30°C. The positive and negative controls were grown on each plate. To each plate 8 ml of the reaction mix was added.

For 25 ml of the reaction mix

0,175 g Agar

17,5 ml Water

5 ml 1M Tris-HCl, pH 7,4.

The mix was boiled in a microwave and then allowed to cool to 55° C in a water bath. To this 2,5 ml of freshly prepared APNE in Dimethyl Formamide (at a concentration of 2 mg/ ml) was added. Immediately before assay 20 mg of Fast Garnet GBC salt was added. The mix was vortexed at maximum speed for a short period. 8 ml of the mix was added to each plate and allowed to solidify. The colour development was observed over a period of time.

3.3.11 Ape1p activity assay

Vacuoles were isolated using the protocol described in 3.3.7.1. The vacuoles in the 0-4 % interface of the ficoll gradient were collected and vortexed for 30 secs at maximum speed.

In the reaction cuvette the following buffer was taken

0,9 ml of 75 mM Tris/ Cl pH 7,5 and 3 mM EDTA

0,1 ml of 10 mM Zinc chloride

0,075 ml of H-Leu pNA (5,03 mg/ ml in DMSO)

The buffer was allowed to reach 30°C. Then 15 µg of the vortexed vacuoles made up to a final volume of 425 µl was added to it. 0,425 ml of 4 % ficoll in 200 mM sorbitol and 10 mM PIPES 6,8 in the reaction buffer was taken as the blank. The absorbance at 405 nm was measured immediately and

then after every 5 min for 15 min. To the reaction mix 50 % TCA was added to a final concentration of 10 %. The precipitated proteins were then washed with cold acetone and solubilised in 1x Laemmli buffer. The proteins were separated by SDS PAGE and the western blot analysis was done using an antibody against Ape 1. The signals detected were quantified using WINCAM and the specific vacuolar Ape1p activity was calculated.

3.3.12 Immunofluorescence Microscopy

Cells were grown to the exponential phase at 25°C. Cells were pelleted at 2500xg for 2 min. The pellet was resuspended in fixative, 3.5 % Paraformaldehyde, 10 % Sorbitol/ PBS, and incubated for 2 hours at room temperature. Cells were washed with PBS/ 10% Sorbitol. Spheroplasting was done in 100 µl PBS/ 10 % Sorbitol with 0.14 µl β-Mercaptoethanol and Lyticase for 1 hour at 30°C. The cells were washed with PBS/ 10 % Sorbitol and adsorbed to multiwell slides treated with poly-L-Lysine. Cells were quenched with PBS/ 10 % Sorbitol/ 1 % BSA for 10 min at room temperature. The cells were then incubated with antibodies against Ssa1p and Ssa2p diluted 1:4000 in quenching solution. After treatment with the primary antibody cells were washed five times with PBS/ 10 % Sorbitol. Secondary Cy3-conjugated goat anti rabbit antibody in quenching solution was added for 30 min. Cells were quickly rinsed, washed twice for 5 min in PBS/ 10 % Sorbitol. The cells were then washed quickly three more times in the same buffer. Embedding medium supplemented with 0.1 µg/ ml DAPI was added and slides were covered with cover slips.

4. RESULTS

4.1 Accumulation of pApe1p in the *ssaI* and *ssaII* deletion strains

Strains lacking Ssa1p or Ssa2p or both were used to determine if the cytosolic HSP70s play a role in the transport of pApe1p to the vacuole. The precursor form of Ape1p (pApe1p) has an apparent molecular weight of 61 kDa as detected by reducing SDS-PAGE. Appearance of the 50 kDa mature Ape1p (mApe1p) indicates that the protein has been transported into the vacuole since maturation of the protein can occur only within the vacuole.

HSP70 dependance of pApe1p transport was followed under varying growth conditions. This was done to determine if the constitutively expressed or the stress induced Ssa proteins or both are involved in the transport of pApe1p to the vacuole.

4.1.1 Levels of precursor and mature form of Ape1p at normal growth temperatures

Two members of the *SSA* subfamily of cytosolic HSP70s, *SSAI* and *SSAII*, are constitutively expressed in the cell, where as *SSAIII* and *SSAIV* are stress induced.

The *Δ ssaI*, *Δ ssaII*, *Δ ssaI/II* and the isogenic wild type strain were grown to the mid log phase at the normal growth temperature of 30°C in nutrient rich media (YPD). Cells were spheroplasted and lysed using glass beads as described in 3.3.2.1. 70 µg protein from each cell type was separated by SDS-PAGE and the western blot analyses were done using antibodies against Ape1p and hexokinase. The signals obtained were quantified using WinCam.

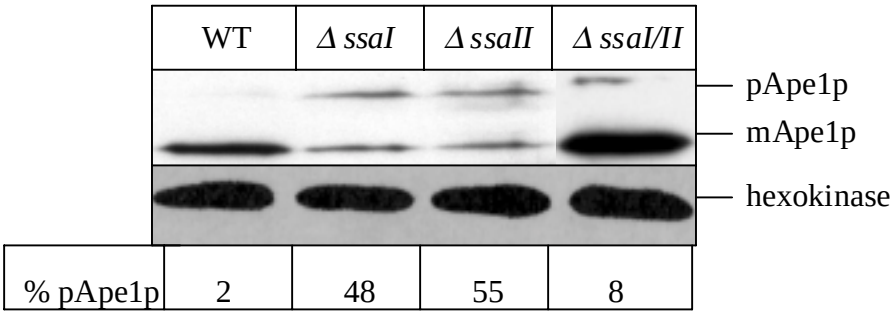


Fig.2: Western blot analyses of cell extracts from the *ssa* deletion strains and the isogenic wild type using antibodies against Ape1p and hexokinase, which served as an internal control. The HRP tagged goat anti-rabbit secondary antibody was used and signals were detected using the super signal chemiluminiscent substrate from PIERCE.

Western blot analysis with Ape1p antibody (Fig.2, upper panel) shows that in the wild type (WT) strain only the mature form of Ape1p (mApe1p) can be seen. In the *ssaI (ΔssaI*) and *ssaII (ΔssaII)* single knock-out cells we see an accumulation of the precursor form of Ape1p (pApe1p). In addition to the accumulated pApe1p, mApe1p is also seen. Interestingly, in the *ssaI/II (ΔssaI/II)* double knock-out, instead of an enhanced phenotype, a decrease in the pApe1p accumulation is seen when compared to the accumulation levels detected in the single knock-out strains. In the double knock-out pApe1p accounts for 8 % of the total Ape1p detected while in the *ssaI* and *ssaII* single knock-outs it accounts for 48 % and 55 % respectively. The phenotype observed in the *ΔssaI/II* cells can be explained by the induced expression of Ssa3p and Ssa4p in the double knock-out under normal growth conditions (Fig. 6, Section 4.3.1).

In the *ΔssaI* and *ΔssaII* cells a decrease in the total levels of Ape1p is seen when compared to the levels in the wild type cells. However, the Ape1p expression is upregulated when the mutant cells are heat shocked at 36°C (Fig.

3, Section 4.1.2). Heat stress is known to increase the expression levels of most vacuolar proteins (Gross and Schulz-Harder, 1986). An increase in the Ape1p expression levels under heat shock conditions, in the *ΔssaI* and *ΔssaII* strains to wild type levels, suggests that the reduced levels in the single knock-out strains grown at 30°C is an indirect effect of the absence of Ssa proteins. Further, deleting proteinase A in the *ΔssaI* strain did not increase the amount of Ape1p detected (Fig. 13, Section 4.5.3). This excludes the possibility of an increased vacuolar turn-over of the wrongly assembled or mis-folded Ape1p.

Western blot analysis with the hexokinase antibody (Fig.2, Lower panel) shows that similar amounts of protein were analysed in each lane.

The accumulation of pApe1p in the *ssa* deletion strains indicates that the cytosolic HSP70s might have a role in the cytosol to vacuole targeting of the protein.

4.1.2 Levels of pApe1p and mApe1p under heat shock conditions

It has been suggested that the four Ssa proteins have overlapping functions and that the overall amount of Ssa proteins may be responsible for a certain effect (Werner-Washburne *et al*., 1987). Under conditions in which some of the SSA family members are over expressed the loss of another family member can be compensated. *SSAIII* and *SSAIV* are stress induced. So we wanted to determine if the over expression of Ssa3p and Ssa4p, under heat shock conditions, can take over the transport of pApe1p.

The wild type, *ssaI* and *ssaII* single knock-outs and the *ssaI/II* double knock-out were grown to the mid log phase at 30°C. Cells were then heat shocked at 36°C for 1 hour. Cells were lysed using glass beads (3.3.2.1). 70 μg protein from each cell type was separated by SDS-PAGE and the western blot analyses were done using antibodies against Ape1p and hexokinase, which was used as an internal control. The signals obtained in the two western blots were

then quantified using WinCam. The pApe1p to mApe1p ratio detected in each strain was then normalised with the levels of the, internal control, hexokinase detected.

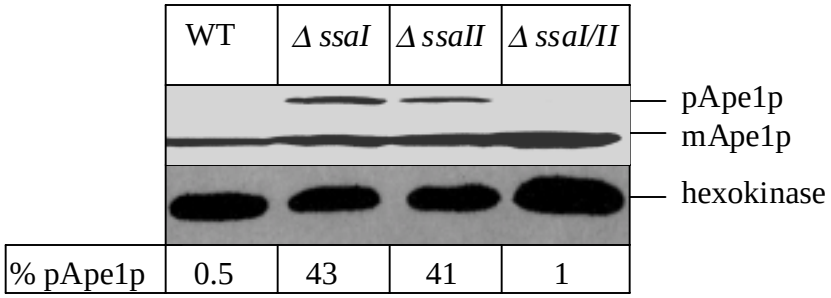


Fig.3: Western blot analyses of the heat shocked *ssa* deletion strains and the wild type cells using antibodies against Ape1p and hexokinase. Goat anti-rabbit secondary antibody tagged to HRP was used. The super signal chemiluminiscent substrate from PIERCE was used to detect the signals.

Western blot analysis with the Ape1p antibody (Fig.3, upper panel) shows that in the wild type strain (WT) only the mature form of Ape1p (mApe1p) is seen. In the *ΔssaI* and *ΔssaII* single knock-out strains an accumulation of the precursor form of Ape1p (pApe1p) is seen, in addition to the mApe1p. However, in the *ssaI/II* double knock-out *(ΔssaI/II*) under heat shock conditions no pApe1p accumulation can be detected. The double knock-out strain now exhibits a wild type phenotype. This result supports the idea that Ssa proteins might have a role in pApe1p transport. It also suggests that under conditions of heat shock, Ssa3p and Ssa4p might be expressed to a level that they can functionally replace Ssa1p and Ssa2p, in a double knock-out, for the pApe1p transport (Fig.7, Section 4.3.2).

In the *ssaII* single knock-out cells also Ssa3/4p are expressed under heat shock conditions (Fig.7, Section 4.3.2). However, the expression level is not sufficient to take over the pApe1p transport function completely (Fig.10, Section 4.4).

It is interesting to note that in the *ΔssaII* cells grown at normal growth temperatures 55 % of the total Ape1p detected is in the precursor form (Fig.2, Section 4.1.1) while under heat shock conditions only 41 % of the total Ape1p is in the precursor form (Fig.3, Section 4.1.2). In the *ΔssaI* cells however, a significant difference is not detected (48 % pApe1p under normal growth temperatures and 43 % under heat shock conditions). This might be because of an induction of *SSAI* under heat shock conditions, in the *ΔssaII* cells. It is known that *SSAI* is induced under heat stress while *SSAII* is not (Werner-Washburne *et al*., 1987).

Western blot analysis with the hexokinase antibody (Fig.3**,** second panel) shows that similar amounts of protein were detected in each lane.

4.2 Dependance of Ape1p transport on Ssa1p and Ssa2p

4.2.1 Complementation by the *SSA* genes

To confirm the involvement of Ssa proteins in the transport of pApe1p to the vacuole a single copy plasmid containing the genomic locus of about 5.5 kb of the *SSAII* gene under its endogenous promoter was introduced into the *ΔssaII* cells. Cells were grown to the mid log phase at the normal growth temperature of 30°C in nutrient rich media (YPD). Cells were spheroplasted and lysed using glass beads (3.3.2.1). 70 µg protein from each cell type was separated by SDS-PAGE and western blot analysis was done using the Ape1p antibody. The signals obtained were quantified by WinCam.

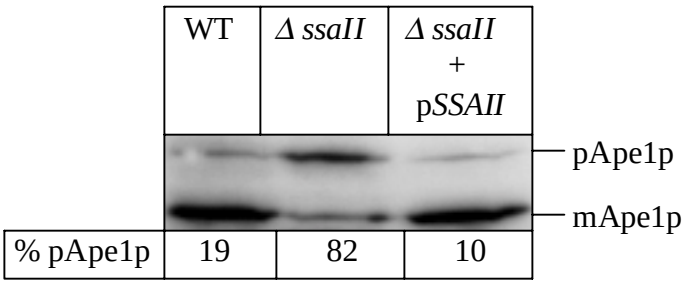


Fig.4: Western blot analysis of the wild type, *ΔssaII* and the *ΔssaII* cells transformed with the *SSAII* gene, using an antibody against Ape 1p. Goat anti-rabbit secondary antibody tagged to HRP was used and the signals were detected using the super signal chemiluminiscent substrate from PIERCE.

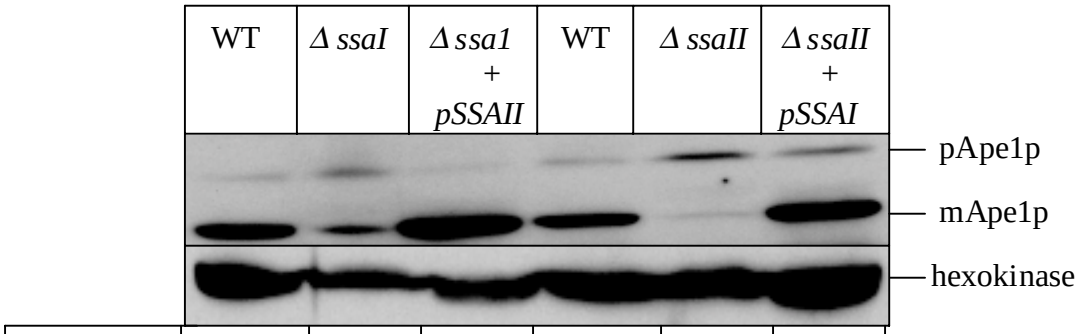
Wild type (WT) cells contain mainly the mature form of Ape1p, as shown by the western blot analysis. 81 % of the total Ape1p detected, in the wild type cells, is the 50 kDa mApe1p while pApe1p accounts for the remaining 19 %. In the *ΔssaII* cells however, an accumulation of the precursor form of the protein (pApe1p) is seen. mApe1p is also detected in these cells, but the pApe1p to mApe1p ratio in the *ΔssaII* cells is much higher (82 % pApe1p and 18 % mApe1p) compared to that seen in the wild type (WT) cells (19 % pApe1p and 81 % mApe1p). Expression of the *SSAII* gene, in a single copy plasmid under the control of its endogenous promoter, in the *Δ ssaII* cells (*ΔssaII + pSSAII*) shows a wild type phenotype. 90 % of the total Ape1p detected is mApe1p while pApe1p corresponds to 10 %, in the transformed cells.

Recovery of pApe1p transport in the *ΔssaII* strains on expressing *SSAII*, from a single copy plasmid, confirmed the involvement of Ssa proteins in pApe1p transport.

4.2.2 Over expression of Ssa1p in the *ΔssaII* cells and Ssa2p in the *ΔssaI* cells

The pApe1p transport phenotype seen in the *ΔssaI* and *ΔssaII* cells under normal growth conditions, (Fig.2, Section 4.1.1), suggests that both the Ssa1p and Ssa2p are required for the transport process. So the question arises whether Ssa1p and Ssa2p have overlapping functions or whether they are involved in different steps of the transport process. To determine this, the *ΔssaI* cells were transformed with a single copy plasmid containing the *SSAII* gene under its endogenous promoter. Similarly, the *ΔssaII* cells were transformed with a single copy plasmid containing the *SSAI* gene under its endogenous promoter.

Cells were grown to the mid log phase at the normal growth temperature of 30°C in rich media (YPD). Cells were spheroplasted and lysed using glass beads (3.3.2.1). Proteins were solubilised in 1x Laemmli buffer. 70 μg protein from each cell type was separated by SDS-PAGE and the western blot analyses were done using antibodies against Ape1p and hexokinase, which was used as an internal control. The signals obtained with the hexokinase control and the Ape1p western were quantified using WinCam. The levels of pApe1p and mApe1p detected were then normalised with the levels of hexokinase detected.



% pApe1p	5	49	2	10	98	12

Fig.5: Western blot analyses of the WT, *Δ ssaI*, *ΔssaI* over expressing Ssa2p, *ΔssaII* and *ΔssaII* over expressing Ssa1p using antibodies against Ape1p and hexokinase. Goat anti-rabbit secondary antibody tagged to HRP was used and the detection was done using the Luminol detection system.

Western blot analysis with the hexokinase antibody (Fig.5. Lower panel), indicates that similar amounts of protein were detected in each lane.

Western blot analysis with the aminopeptidase 1 antibody (Fig.5. upper panel) shows that in the wild type cells (WT) only the mature form of Ape1p (mApe1p) is seen. In the *Δ ssaI* and *Δ ssaII* single knock-out cells an accumulation of the precursor form of the protein (pApe1p) is seen. mApe1p is also detected in these cells. In the *Δ ssaI* cells over expressing Ssa2p (*Δssa1 + pSSAII*) only mApe1p can be seen. Similarly, in the *ΔssaII* cells over expressing Ssa1p (*ΔssaII + pSSAI*), we do not see an accumulation of pApe1p (12 % pApe1p and 88 % mApe1p) as seen in the untransformed (*ΔssaII*) cells (98 % pApe1p and 2 % mApe1p). In these cells mainly mApe1p is seen. The pApe1p to mApe1p ratio in the transformed (*ΔssaI + pssaII* and *ΔssaII + pssaI*) cells is similar to that seen in the wild type cells (WT: 5 % pApe1p and 95 % mApe1p; *ΔssaI + pSSSAII*: 2 % pApe1p and 98 % mApe1p; *ΔssaII + pSSAI*:12 % pApe1p and 88 % mApe1p).

Wild type phenotype seen in the *ΔssaI + pSSAII* and *ΔssaII + pSSAI* cells indicates that the Ssa1p and Ssa2p have overlapping functions in the transport of pApe1p to the vacuole. Block in the transport of pApe1p would have persisted if the two proteins were involved in different steps of the transport process.

4.3 Dependance of Ape1p transport on Ssa3/4p

4.3.1 Expression of Ssa3/4p at normal growth temperatures

Accumulation of pApe1p is observed when the *ssaI/II* double knock-out is grown at 30°C (Lane: *ΔssaI/II* in Fig. 2, Section 4.1.1). However, the amount of pApe1p does not increase when compared to the levels seen in the *ssaI* and *ssaII* single knock-out strains (Lane: *ΔssaI* and *ΔssaII* in Fig. 2, Section 4.1.1), but is decreased. This might be caused by an increase in the expression of Ssa3/4p, whose expression is normally induced only under heat shock conditions. So the expression levels of Ssa3/4p in the wild type and the respective *ssa* deletion strains were determined.

The *ΔssaI*, *ΔssaII*, *ΔssaI/II* and the isogenic wild type (WT) strain were grown to mid log phase at the normal growth temperature of 30°C in nutrient rich media (YPD). Cells were lysed using glass beads as described in 3.3.2.1 and 70 μg protein from each cell type was separated by SDS-PAGE and the western blot analyses were done using an antibody which detects Ssa3/4p and an antibody against hexokinase. Ssa3p and Ssa4p are 97 % homologous and the antisera does not discriminate between the two proteins. Western blot analysis with the hexokinase antibody was done as an internal control.

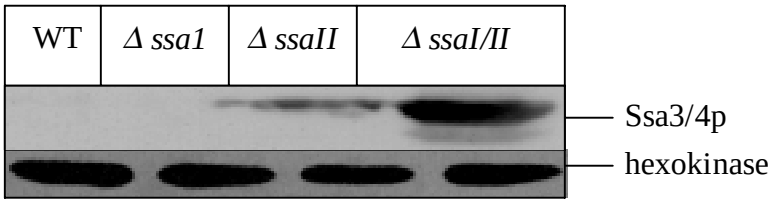


Fig.6: Western blot analyses of the cell extracts from the *ΔssaI, ΔssaII, ΔssaI/II* and the isogenic wild type using antibodies against Ssa3/4p and hexokinase. The HRP tagged goat anti-rabbit secondary antibody was used and the detection was done using the super signal chemiluminiscent substrate from PIERCE.

Western blot analysis with the Ssa3/4p antibody (Fig.6, upper panel) shows that in the wild type (WT) and the *ΔssaI* cells there is no expression of Ssa3p and Ssa4p at the normal growth temperatures. In the *Δ ssaII* cells low levels of Ssa3/4p can be detected. Under normal growth conditions the cytosol contains about 70 % Ssa2p and 30 % Ssa1p. Since Ssa2p is the major constitutively expressed HSP70, in its absence expression of Ssa3p and Ssa4p is upregulated. In the *ΔssaI/II* cells however, there is a 4-5 fold increase in the expression of Ssa3/4p when compared to the expression in the *ΔssaII* cells (Refer Fig. 6 and Fig.10). This shows that Ssa3/4p takes over the function of pApe1p transport in the *ssaI/II* double knock-out even at the normal growth temperature (Compare Lanes: *ΔssaI* and *ΔssaII* with *ΔssaI/II* in Fig.2, section 4.1.1). It also confirms data in the literature, that the four Ssa proteins perform overlapping funtions.

Western blot analysis with hexokinase antibody (Fig.6, Lower panel) shows that similar amounts of protein were detected in each lane. The signals detected in the two westerns were quantified using WinCam and then normalised for determining the Ssa3/4p expression levels in the different strains.

4.3.2 Ssa3/4p expression under heat shock conditions

Two members of the SSA subfamily, Ssa3p and Ssa4p are expressed normally only under heat shock conditions. However, in the *ΔssaI/II* cells Ssa3p and Ssa4p are expressed at normal growth temperatures also (Fig. 6, Section 4.3.1) and they can partially take over the function of Ssa1/2p in the transport of pApe1p to the vacuole (Fig. 2, Section 4.1.1). Under heat shock conditions the *ΔssaI/II* cells exhibit a wild type phenotype for pApe1p transport (Fig. 3 Section 4.1.2). So in the *ΔssaI/II* cells, under heat shock conditions, Ssa3/4p might be massively overexpressed compared to the levels expressed under normal growth temperatures. Hence the expression levels of Ssa3/4p in the wild type, *ΔssaII* and *ΔssaI/II* were determined.

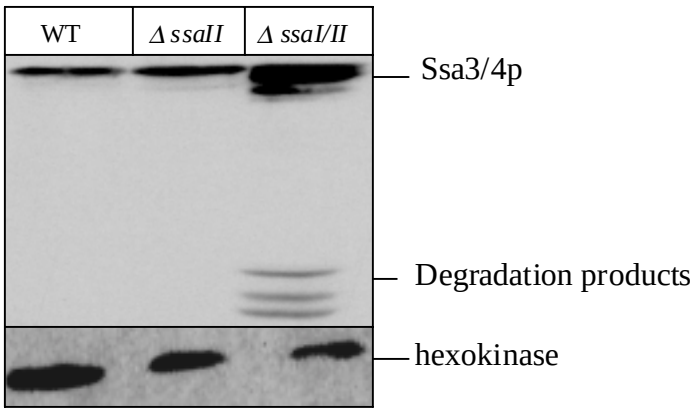


Fig.7: Western blot analyses of the *ssa* deletion strains and the wild type cells under heat shock conditions using antibodies against Ssa3/4p and hexokinase. The HRP tagged goat anti-rabbit secondary antibody was used and the detection was done using the super signal chemiluminiscent substrate from PIERCE.

The *ssaII* single knock-out, *ssaI/II* double knock-out and the isogenic wild type strain were grown to the mid log phase in nutrient rich media (YPD)

at the normal growth temperature of 30°C. Cells were then heat shocked at 36°C for 1 hour. Cells were spheroplasted and lysed using glass beads (ref. 3.3.2.1). 70 μg protein from each cell type was separated by SDS-PAGE. Western blot analysis with the Ssa3/4p antibody (Fig.7, upper panel) shows that in the wild type (WT) and the *ΔssaII* cells the expression level of Ssa3/4p is similar (also shown in the bar diagram in Fig.10). However, in the *ΔssaI/II* double knock-out there is a 4 fold increase in the levels of the Ssa3/4p when compared to the levels in the wild type and *ΔssaII* cells (Fig.7, also refer Fig.10). An over expression of Ssa3/4p in the *ΔssaI/II* double knock-out takes over the function of pApe1p transport, under heat shock conditions (Lane *ΔssaI/II* in Fig.7, Section 4.3.2 and Lane *ΔssaI/II* in Fig.3, Section 4.1.2), showing that the Ssa proteins have overlapping functions. As shown in the bar diagram in Fig.10, Ssa3/4p expression levels in the *ΔssaI/II* cells under heat shock conditions is nearly two times the levels detected under normal growth conditions suggesting that it is the final levels of the Ssa proteins, in the cytosol, which affects pApe1p transport.

Western blot analysis done with the hexokinase antibody (Fig.7, lower panel) shows that similar amounts of protein were detected in each lane. The signals obtained by the anti-Ssa3/4p and anti-hexokinase antibodies were quantified using WinCam and then normalised for determining the expression levels of Ssa3/4p in the different strains.

4.4 Requirement of Ssa proteins for autophagocytosis

It has been suggested that the vesicle mediated transport of pApe1p to the vacuole is mechanistically similar to the transport of cytosolic proteins and organelles to the vacuole by autophagocytosis. Many of the components required for the cytosol to vacuole targeting of pApe1p are also required for

autophagocytosis. Therefore we tested if autophagocytosis is impaired by Ssap deficiency.

Wild type and *ΔssaII* cells were grown to the mid log phase in nutrient rich media. To induce autophagy, *ΔssaII* cells were grown to the stationary phase in YPD (cells were grown for 48 hours till they reached $O.D._{600}$ 2.0) or to mid log phase ($O.D._{600}$ 0.8) in nitrogen starvation media (SD-N). Cells were spheroplasted using Zymolyase 20T (3.3.1) and lysed using glass beads (3.3.2.1). Cell extracts were boiled in Laemmli buffer (3.3.3.3) and separated by SDS-PAGE. Western blot analysis was done using the Ape1p antibody.

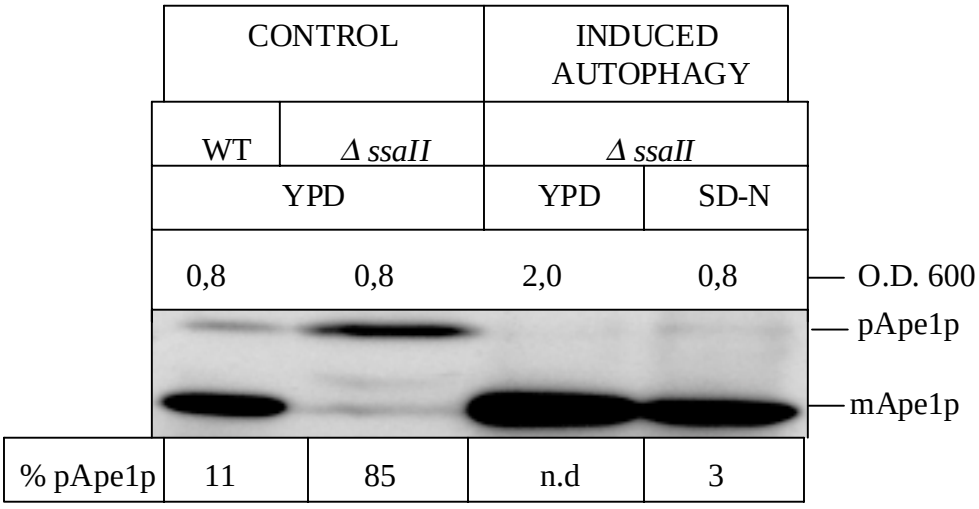


Fig.8: Western blot analysis, of the Wild type and *ΔssaII* cells grown in YPD or in autophagy inducing media, using an antibody against Ape1p. Goat anti-rabbit secondary antibody tagged to horse radish peroxidase was used. Signals were detected using the Luminol detection system.
n.d Not detected

When wild type cells (WT) were grown to the mid log phase ($O.D._{600}$ 0.8) in nutrient rich media (YPD) mature form of Ape1 (mApe1p) could be detected. In these cells pApe1p corresponds to about 11 % of the total Ape1p detected. pApe1p accumulated in the *ΔssaII* cells grown to the mid log phase in

YPD media (*ΔssaII*, YPD, $O.D._{600}$ 0.8), as demonstrated previously (Fig.2, Section 4.1.1). However, no pApe1p accumulation was observed in the *ΔssaII* cells under autophagy conditions. *ΔssaII* cells exhibited a wild type phenotype when grown to the stationary phase in YPD (*ΔssaII*, YPD, $O.D._{600}$ 2.0) or to mid log phase in nitrogen starvation media (*ΔssaII*, SD-N, $O.D._{600}$ 0.8).

The above experiment shows that induction of autophagy, by glucose depletion or nitrogen starvation, can take over the transport of pApe1p to the vacuole in the *ssa* deletion strains.

As seen previously (Lane: *ΔssaI/II*, Fig.7, section 4.3.2 and Lane: *ΔssaI/II*, Fig.3, Section 4.1.2), an over expression of Ssa3/4p under conditions of heat stress can take over pApe1p transport completely. Therefore we tested whether over expression of Ssa3/4p in the *ΔssaII* cells, under autophagy conditions, explains reversal of the phenotype.

The *ΔssaII* cells were grown in rich media to mid log phase or autophagy was induced in these cells by growing them in YPD to the stationary phase or by growing them in nitrogen starvation media. Wild type cells were grown to the mid log phase in nutrient rich media. Cells were spheroplasted (3.3.1) and lysed using glass beads (3.3.2.1). Proteins were boiled in Laemmli buffer (3.3.3.3) and separated by SDS-PAGE and the western blot analysis was done using an antibody against Ssa3/4p.

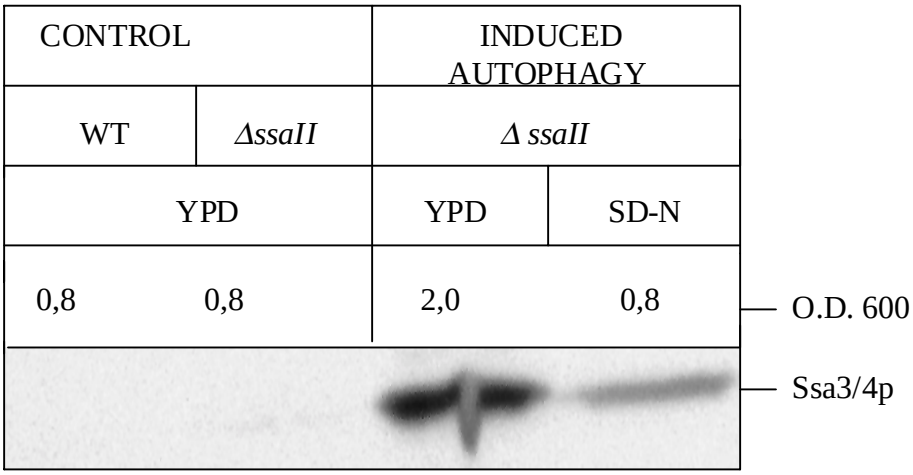


Fig.9: Western blot analysis, of the cell extracts from the wild type and *ΔssaII* cells grown in nutrient rich media or in autophagy inducing media, using the anti-Ssa3/4p antibody. HRP tagged goat anti-rabbit secondary antibody was used. Signals were detected using the Luminol detection system.

Ssa3/4p could not be detected in the wild type (WT) or *ΔssaII* cells when they were grown to the mid log phase in rich media (WT, YPD, $O.D._{600}$ 0.8 and *ΔssaII*, YPD, $O.D._{600}$ 0.8). When autophagy was induced, by growing the *ΔssaII* cells to stationary phase in YPD media (*ΔssaII*, YPD, $O.D._{600}$ 2.0) or by growing them to mid log phase in nitrogen deficient media (*ΔssaII*, SD-N, $O.D._{600}$ 0.8), Ssa3/4p could be detected.

The levels of Ssa3/4p expressed in the *ΔssaI/II* cells under heat shock conditions can take over pApe1p transport completely. So are the levels of Ssa3/4p expressed under heat shock conditions and nutrient limiting conditions similar? The Ssa3/4p signals seen in the *ΔssaII* cells under autophagy conditions were quantified. This was compared to the levels of Ssa3/4p expressed in the *ssa* deletion strains under normal growth temperatures (Refer Fig.6, Section 4.3.1) and under heat shock conditions (Refer Fig.7, Section 4.3.2).

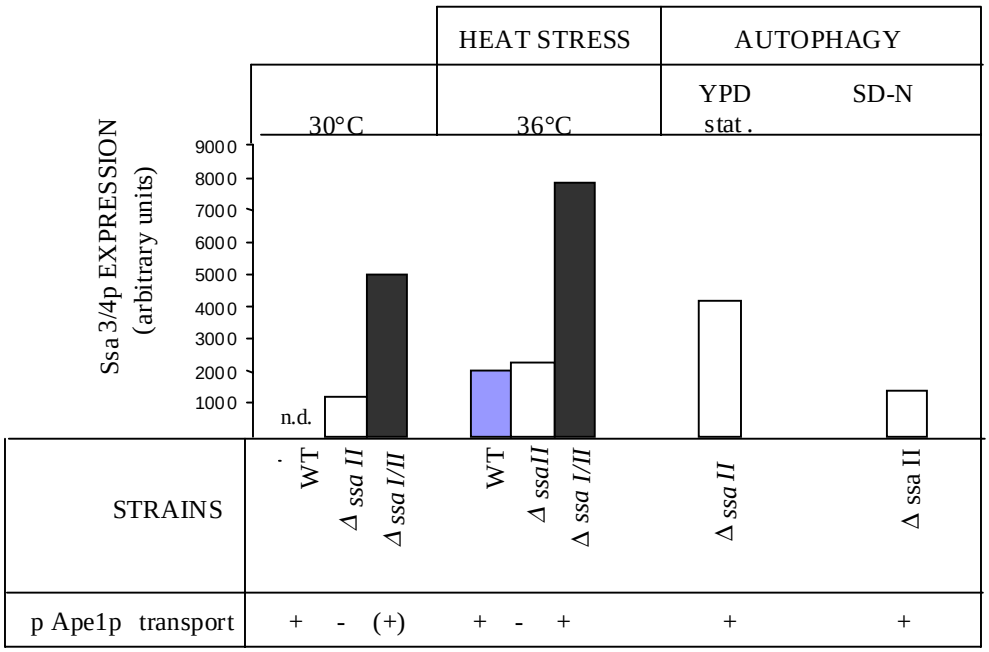


Fig.10: Comparison of the levels of Ssa3/4p expressed in the *ssa* deletion strains under heat stress and under autophagy conditions.
The Ssa3/4p signals obtained in the western blots shown in **Fig.6**, Section 4.3.1, **Fig.7**, Section 4.3.2 and **Fig.9**, Section 4.4 have been quantified and compared.
n.d: Not Detected.
(+): Not complete transport as in wild type cells.

The Ssa3/4p expression level in the *ΔssaII* cells under nitrogen starvation conditions (SD-N) is as high as the expression in the *ΔssaII* cells grown at 30°C. An accumulation of pApe1p was seen in the *ΔssaII* cells grown at 30°C (Fig. 2, Section 4.1.1) indicating that the Ssa3/4p expression levels are not sufficient to take over the pApe1p transport. However, in the *ΔssaII* cells grown under nitrogen starvation conditions at 30°C no pApe1p accumulation was seen (Fig. 8, Section 4.4). The Ssa3/4p expression level in the *ΔssaII* cells grown to stationary phase in nutrient rich media is lower than the expression in the *ΔssaI/II* at 30°C, where the Ssa3/4p levels could not restore the pApe1p transport completely. Further, the levels of Ssa3/4p expressed in the *ΔssaII* cells

under autophagy conditions is 2-4 times less compared to the expression in *ΔssaI/II* at 36°C, when pApe1p transport is completely restored. Therefore, pApe1p transport under starvation conditions occurs independently of Ssa3/4p.

So cytosolic HSP70s appear to play a role in the cytosol to vacuole targeting of pApe1p and not in the autophagy mediated transport of the protein to the vacuole.

4.5 Oligomeric status of the accumulating pApe1p in the *ssa* deletion strains

A decrease in the levels of cytosolic HSP70s leads to an accumulation of the precursor form of Ape1p. The cytosolic HSP70s might be assisting in the oligomerisation of the protein. Glycerol density gradients were used to determine the oligomeric state of the accumulating precursor.

4.5.1 Glycerol Density gradients

The *ssaII* knock-out and the isogenic wild type cells were grown to the mid log phase in rich media (YPD) at 30°C. Cells were spheroplasted and then lysed using glass beads (3.3.2.1). Cell lysate was loaded on top of a glycerol density gradient and centrifuged for 4 hours at 55000 rpm (260000xg) in a Beckman table top ultracentrifuge (3.3.6). The fractions were TCA precipitated and solubilised in 1x Laemmli buffer. Proteins were separated by SDS-PAGE and the western blot analysis was done using an antibody against Ape1p.

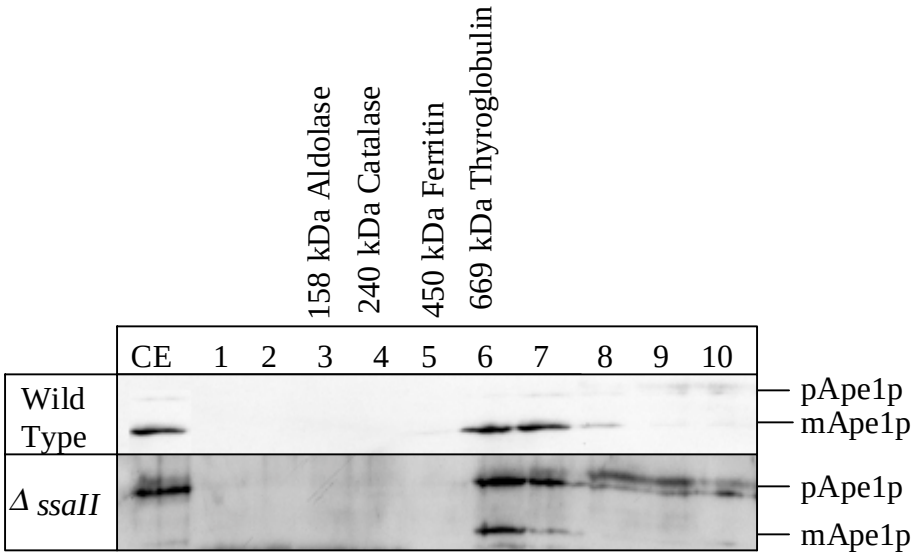


Fig.11: Western blot analysis of fractions collected from the glycerol density gradients, using an antibody against Ape1p. HRP tagged goat anti-rabbit secondary antibody was used and the detection was done using the super signal chemiluminiscent substrate from PIERCE.
Molecular weight markers mentioned were also separated on a glycerol gradient and detected by Coomassie blue staining.

In the first panel in Fig.11 is the western blot analysis of the starting material and the fractions collected from the gradient containing wild type cells. The first lane, labelled CE (cell extract), is an aliqout of the lysed cells which was loaded on the glycerol density gradient. The 600 kDa dodecameric mature Ape1p (mApe1p) appears mainly in the 6^{th} and 7^{th} fractions of the gradient along with the 669 kDa thyroglobulin marker.

The second panel in Fig.11 shows the western blot analysis of the starting material and the fractions collected from the gradient containing the *ΔssaII* cell lysate. The cell extract loaded on the gradient (CE in the second panel) contains mainly the precursor form of Ape1p. The accumulating pApe1p cofractionates with the dodecameric mature form of Ape1p. The precursor and mature form of the protein are seen mainly in the 6^{th} and 7^{th} fractions of the gradient along with the 669 kDa thyroglobulin marker. No precursor Ape1p can

be detected in the earlier fractions of the gradient. This indicates that the accumulating pApe1p in the *ssa* deletion strains is already assembled into a dodecamer. Some pApe1p can be detected in fractions 8, 9 and 10 which might be due to the formation of aggregates.

4.5.2 Generation of the *ΔssaI* and *ΔssaIΔpep4* strain (APNE assay)

The glycerol density gradients showed that the accumulating pApe1p in the *ssa* deletion strain is assembled into a dodecamer. However, the pApe1p dodecamer may be wrongly assembled and as a result might be degraded in the vacuole by the resident proteases. Therefore, a *ΔssaI* strain lacking proteinase A, which is required for the activation of the other vacuolar proteases, was generated. In this strain autophagic bodies would accumulate in the vacuole.

The *ΔssaI* cells were transformed with the PTS15 plasmid. This plasmid contains the *pep4* (encoding proteinase A) knock out construct with uracil as the auxotrophic marker (Ammerer *et al.*, 1986).

Transformants were selected on uracil deficient plates and then grown on YPD media plates. A positive and negative control were also grown on each plate. APNE assay mix was poured on the plates so that a uniform thin layer was formed over the cells (3.3.10). The plates were incubated at room temperature for 10-15 min. The *pep4* knock-out cells remain white where as the negative clones turn red in colour.

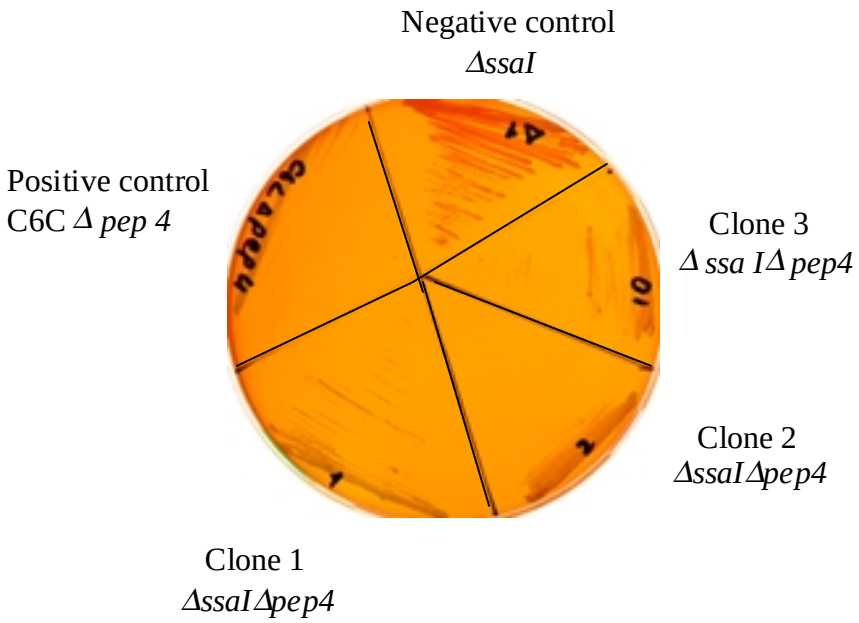


Fig.12: APNE assay to select a *ΔssaIΔpep4* strain. *C6CΔpep4* is used as the positive control while the *ΔssaI* cells are used as the negative control.

As shown in Fig.12, the C6C*Δpep4* strain and the *ΔssaIΔpep4* clones 1, 2 and 3 are white indicating they are the *pep4* knock-out strains. The non-transformed *ΔssaI* strain is red indicating that the *PEP4* gene is intact.

4.5.3 Levels of pApe1p and mApe1p in the *ΔssaI* and *ΔssaIΔpep4* strains

In the *Δssa* strain the accumulating pApe1p is assembled into a dodecamer, as is shown in Fig.11, Section 4.5.1. However, the protein might be wrongly assembled and hence could be degraded. This degradation would lead to a decrease in the total levels of Ape1p in the *ssa* deletion strains when compared to the levels in the WT cells. In fact about a 29 % decrease in the total levels of Ape1p was seen in the *ΔssaI* and *ΔssaII* strains, grown at 30°C,

when compared to the levels in the wild type cells (Fig.2, Section 4.1.1). However, in a *ΔssaIΔpep4* strain, lacking proteinase A, this wrongly assembled protein would not be degraded in the vacuole and hence an increase in the levels of Ape1p would be detected compared to the levels in the *ΔssaI*.

Wild type, *ΔssaI* and *ΔssaIΔpep4* strains were used to determine the total amount of Ape1p. Cells were spheroplasted with Zymolyase 20T (3.3.1) and lysed using glass beads (3.3.2.1). Proteins were boiled in 1x Laemmli buffer at 95°C for 10 min. 70 µg protein was separated by SDS-PAGE and western blot analysis was done using an antibody against Ape1p and hexokinase. The signals were then quantified by WinCam.

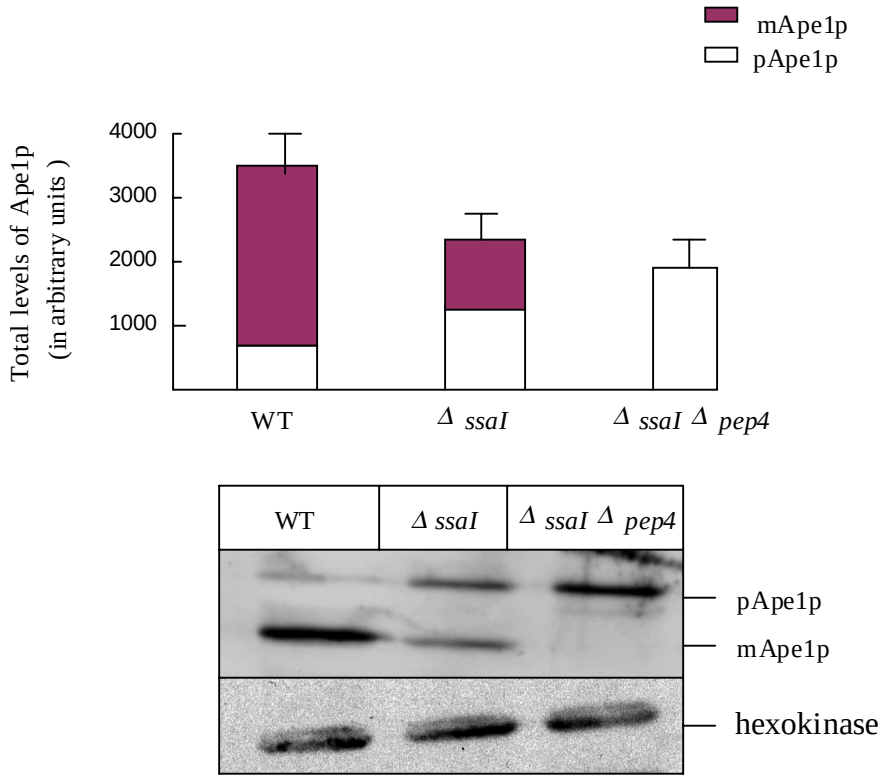


Fig.13: Western blot analysis of the cell lysates from WT, *ΔssaI* and *ΔssaIΔpep4* strain using an antibody against Ape1p and hexokinase. Goat anti-rabbit secondary antibody tagged to HRP was used. The signals were detected using the Luminol detection system.
Signals were quantified using WinCam and are represented as a bar diagram.

As seen in the western blot in Fig.13 there is a 30 % decrease in the total levels of Ape1p detected in the *ΔssaI* strains when compared to the levels in the wild type cells. However, there is no increase in the levels of Ape1p detected in the *ΔssaIΔpep4* cells when compared to the Ape1p levels detected in the *ΔssaI* cells. As seen previously, in the *ΔssaI* cells an accumulation of pApe1p is observed while in *the ΔssaIΔpep4* cells only the pApe1p is seen. The similar levels of Ape1p detected in the *ΔssaI* and *ΔssaIΔpep4* cells suggests that there is no misassembly of the accumulating pApe1p in the *ssa* deletion cells. An increase in the levels of Ape1p in the *ΔssaIΔpep4* when compared to the levels in the *ΔssaI* cells would have indicated that the accumulating dodecameric pApe1p might be wrongly assembled and hence degraded in the vacuole.

The second panel in Fig.13 is the western blot analysis done with the hexokinase antibody which shows that equal amounts of protein were analysed in each lane.

4.5.4 Specific activity of the vacuolar Ape1p in the *ssa* deletion strain.

Similar amounts of Ape1p detected in the *ΔssaI* and *ΔssaIΔpep4* strains indicated that the accumulating pApe1p might be correctly assembled. To confirm this the specific activity of the vacuolar Ape1p in the WT and *ssa* deletion strains was determined.

Cells were spheroplasted using Zymolyase 20T (3.3.1) and lysed using water (3.3.2.3). Cell lysates were separated on ficoll discontinous gradients (3.3.7.1). The gradients were centrifuged at 30000 rpm (160000xg) for 90 min at 4°C in a Beckman SW40 rotor. The 0-4 % interface containing vacuoles was collected. The vacuoles were lysed by vortexing and the specific activity of aminopeptidase I was determined using H-Leu-pNA as the substrate (3.3.11). A fraction of the vacuoles were TCA precipitated. Proteins were boiled in

Laemmli buffer and separated by SDS-PAGE. Western blot analysis was done using antibodies against Ape1p. The Ape1p signals obtained were then quantified by WinCam and used to calculate the specific aminopeptidase I activity.

Strains	**Ape1p activity** $\frac{[\Delta E]}{[10 \text{ min} \times \text{mg protein}]}$	**Ape1p in the Vacuolar fraction** [Pixel counts]	**Specific vacuolar Ape1p activity** $\frac{[\text{Ape1p activity}] \times 10^{-3}}{[\text{pixel count}]}$
WT	0.8791 ± 0.0208	7019.66 ± 500	0.1256 ± 0.0093
ΔssaI	0.2875 ± 0.0125	1417.66 ± 50	0.2029 ± 0.0062
ΔssaII	0.2333 ± 0.0083	2309.50 ± 1300	0.1117 ± 0.0471
ΔssaI/II	2.5833 ± 0.3167	10110 ± 50	0.2749 ± 0.0119

Fig.14: Determining the specific activity of aminopeptidase I in the wild type and *Δssa* cells at 405 nm. Western blot analysis of the vacuoles was done using antibodies against Ape1p. Goat anti-rabbit secondary antibody tagged to HRP was used and the signals were detected using the Luminol detection system.

As shown in Fig.14 the specific activity of Ape1p in the wild type and *ΔssaII* strains is similar. In the *ΔssaI* and *ΔssaI/II* strains however, an increase in the activity is observed. A wrong assembly of the dodecameric Ape1p would have resulted in a decrease in the specific activity of the protein in the *ssa* deletion strains when compared to the activity in the wild type cells. This result shows that there is no defect in the assembly of pApe1p in the *ssa* deletion strains.

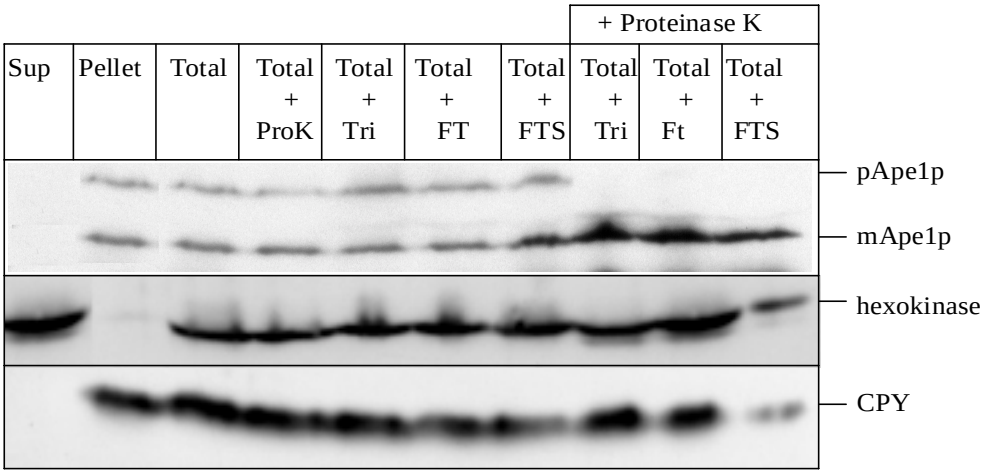
4.6. Localization of the accumulating precursor Ape1p in the *ssa* deletion strains

A decrease in the levels of cytosolic HSP70s causes an accumulation of pApe1p which is already assembled into a dodecamer. Next we determined, where the pApe1p is accumulating in the cell. It could accumulate in the cytosol, in cvt vesicles, or in the vacuole.

4.6.1 Protease protection experiment

To determine if the accumulating dodecameric pApe1p is present in the cytosol or in a membrane bound compartment, Protease protection experiments were done.

ssaII knock-out cells were grown to the mid log phase at 30°C in YPD media. Cells were spheroplasted using Zymolyase 20T (3.3.1). Spheroplasts were differentially lysed using DEAE Dextran (3.3.2.2). Organellar membranes were lysed using triton X-100 (a detergent) or by physical disruption like freeze thaw and freeze thaw sonication (3.3.9). Lysed cells were then treated with proteinase K. A control sample was taken which was treated with proteinase K while the organellar membranes were kept intact. The samples were incubated on ice for 30 min. Proteinase K was inactivated using PMSF and proteins were precipitated using 20 % TCA and 80 % acetone mix. Proteins were separated by SDS-PAGE and the western blot analyses were done using antibodies against Ape1p, hexokinase (cytosolic protein) and carboxypeptidase Y (vacuolar resident protein).



Sup: Supernatant
Tri: Triton X-100
FT: Freezing in liquid nitrogen and thawing at 37°C
FTS: Freezing in liquid nitrogen and thawing by sonication
ProK: Proteinase K
CPY: Carboxypeptidase Y

Fig.15: Western blot analyses of the cell lysates, treated with proteinase K either by retaining the internal membranes intact or after disruption, using antibodies against Ape1p, CPY (organellar marker) and hexokinase (cytosolic marker). The goat anti-rabbit secondary antibody tagged to HRP was used and the detection was done using the Luminol detection system.

ΔssaII cells were differentially lysed and centrifuged at 5000xg for 5 min and the supernatant (sup) and pellet were separated by SDS-PAGE. In lane 1 and 2 of Fig. 15 the lysis control is shown. The accumulating pApe1p and mApe1p are present only in the pellet fraction. The cell lysis is complete as shown by the hexokinase (Fig.15, second panel) and CPY (Fig.15, third panel) control western blots. The cytosolic protein hexokinase is present only in the supernatant while CPY, a soluble vacuolar resident protein, is found exclusively in the pellet fraction. In lane 3 of Fig.15 is shown the total cell lysate (TOTAL) used for the protease protection assay. The accumulated precursor form of Ape1p (pApe1p) and the mature form of Ape1p (mApe1p)

are both seen here. When the cell lysate is treated only with proteinase K the accumulated pApe1p and mApe1p are detected, as shown in lane 4 (TOTAL + ProK). Similarly when the differentially lysed cells are treated only with a detergent (TOTAL + Tri) or are subjected to freeze thaw (TOTAL + FT) or freeze thaw sonication (TOTAL + FTS) then both forms of Ape1p are seen. However, when the cell lysates are treated with triton X-100 and proteinase K then only the mature form of Ape1p (mApe1p) is detected (lane 8) (TOTAL + Tri+ ProK). Similarly, the accumulated precursor Ape1p (pApe1p) is accessible to proteinase K when the cell lysates are subjected to freeze thaw (TOTAL + FT + ProK) or freeze thaw sonication (TOTAL + FTS + ProK). This shows that the accumulated precursor Ape1p which is seen in the *ssa* deletion strains is accessible to proteinase K only when the internal membranes are disrupted. Hence the accumulating precursor Ape1p is present in a membrane enclosed compartment.

4.6.2 Cell fractionation experiments

The protease protection experiment showed that the accumulating dodecameric precursor Ape1p, seen in the *ssa* deletion strains, is present in a membrane bound compartment. The accumulating pApe1p might be present in cytosolic cvt vesicles or in the vacuole. Using density gradient centrifugation the site of accumulation of pApe1p was determined.

4.6.2.1 Ficoll floatation gradients

The *ssaII* deletion strain and the isogenic wild type strain were used for the localization experiments. The cells were spheroplasted using Zymolyase 20 T (3.3.1) and then lysed using water (3.3.2.3). Cell lysates were separated on a

Ficoll discontinuous floatation gradient. The gradients were centrifuged in a Beckman SW40 rotor at 30000 rpm (160000xg) for 90 min at 4°C (3.3.7.1). The fractions were collected and TCA precipitated. Proteins were separated by SDS-PAGE and the western blot analyses were done using antibodies against Ape1p and carboxypeptidase Y (CPY), which was used as the vacuolar marker.

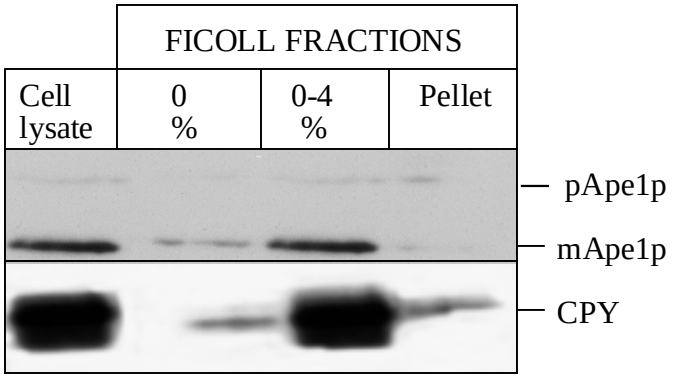


Fig.16a: Western blot analyses of the fractions collected from the Ficoll floatation gradient using antibodies against Ape1p and CPY. HRP tagged goat anti-rabbit secondary antibody was used and the detection was done using the Luminol system.
Cell lysate from wild type cells was loaded on the gradient.

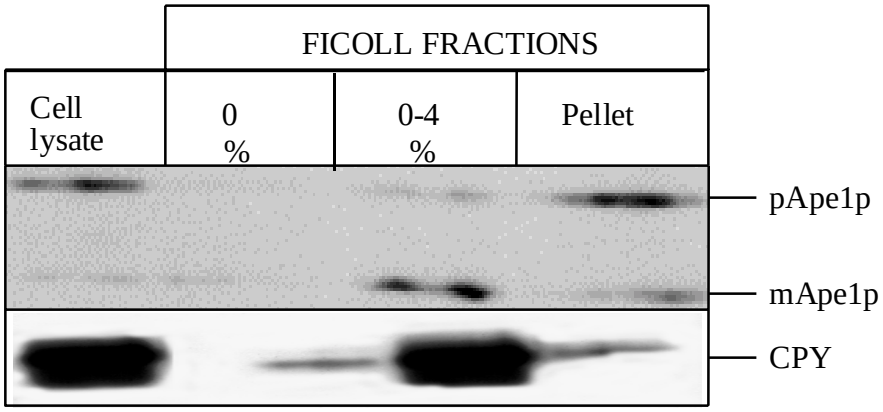


Fig.16b: Western blot analyses of the fractions collected from the Ficoll floatation gradient using antibodies against Ape1p and CPY. HRP tagged goat anti-rabbit secondary antibody was used and the detection was done using the Luminol system. Cell lysate from the *ssaII* deletion strain was loaded on the gradient.

Fig.16a and 16b show the fractions collected from the gradients containing cell lysates from the wild type and *ΔssaII* cells respectively. In both the figures, the upper panel corresponds to the western blot analysis with the Ape1p antibody while the lower panel is the western blot analysis with the CPY antibody.

The first lane indicated as 'cell lysate' is an aliquot of the starting material loaded on the gradient. In the wild type cells (Fig.16a) the mature form of Ape1p (mApe1p) can be seen in the starting material while in the *ΔssaII* cells (Fig.16b) the accumulating precursor Ape1p (pApe1p) is detected. In the mutant and wild type cells mApe1p is present mainly in the vacuolar fraction (0-4 % interface). The 0-4 % interface contains most of the vacuoles as shown by the presence of the CPY marker mainly in this fraction (second panel of both Fig.16a and Fig.16b). mApe1p is detected in the uppermost 0 % fraction also, in case of the wild type cells (Fig.16a). This could be due to the lysis of some of the vacuoles during preparation since the vacuolar marker CPY can also be detected in this fraction. In the wild type cells the small amount of precursor

Ape1p (pApe1p) seen, is present in the pellet of the gradient (Fig.16a). In the *ΔssaII* cells also, the accumulating pApe1p is present mainly in the pellet of the gradient (Fig.16b) and only about 5 % of pApe1p co-fractionates with mApe1p in the vacuolar fraction. mApe1p seen in the pellet fraction (both Fig.16a and Fig.16b) may be due to the unlysed cells and spheroplasts, since CPY can also be detected here. However, pApe1p detected in the pellet of the *ΔssaII* cells containing gradient is much higher than that seen in the pellet of wild type cell lysate containing gradient (compare Pellet fraction in Fig.16a and Fig.16b). This along with the protease protection analysis (Fig.15, Section 4.6.1) shows that the dodecameric pApe1p is accumulating in a membrane bound compartment outside the vacuole.

4.6.2.2 Optiprep sedimentation gradient

In the ficoll floatation gradients it was seen that most of the accumulating pApe1p, in the *Δ ssaII* strain, is not present in the vacuolar fraction. Only about 5% of the accumulating pApe1p co-fractionated with the mature form, while the remaining pApe1p was found in the pellet. In order to confirm that pApe1p, in the *ΔssaII* cells, is accumulating in a compartment outside the vacuole, the *ΔssaII* cell lysate was loaded on an optiprep sedimentation gradient.

Cells were spheroplasted (3.3.1) and lysed using water (3.3.2.3). The cell lysate was loaded on top of an optiprep step gradient and centrifuged at 25000 rpm (111000xg) for 4 hours at 4°C (3.3.8). The fractions were collected and TCA precipitated. Proteins were solubilised in 1x Laemmli buffer and boiled at 95°C for 10 min. Proteins were then separated by SDS-PAGE and western blot analyses were done using antibodies against Ape1p and CPY which was used as the vacuolar marker.

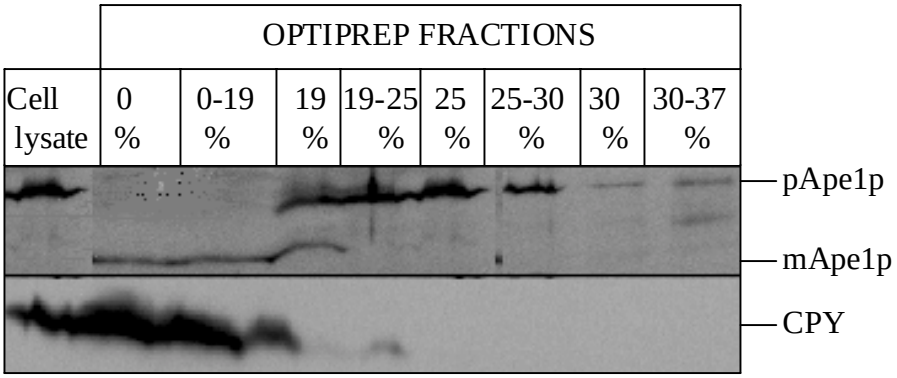


Fig.17: Western blot analyses of the fractions collected from the optiprep sedimentation gradient using antibodies against Ape1p and CPY. Goat anti-rabbit secondary antibody tagged to HRP was used. Signals were detected using the Luminol system

The upper panel in Fig.17 is the western blot analysis done with the Ape1p antibody and the second panel shows the western blot analysis done with the antibody against the soluble vacuolar resident protein CPY. In the figure, the lane labelled 'cell lysate' is an aliquot of the starting material loaded on the gradient. The remaining lanes are the different fractions collected from the gradient. In the starting material an accumulation of the pApe1p is seen. The vacuolar form of Ape1p i.e. the mApe1p is seen mainly in the 0-19 % fraction of the gradient. The vacuolar marker, CPY, is also seen in the same fraction (0-19 %, second panel). mApe1p seen in the 0 % fraction could represent some of the vacuoles which were disrupted during preparation, since CPY is also detected in this fraction. Most of the accumulating pApe1p is seen in the denser regions of the gradient. The accumulating pApe1p is present mainly in the 19-25 % interface and 25 % fraction of the gradient. The vacuolar resident protein CPY is, however, not detected in this fraction. This shows that the pApe1p, in the *ssa* deletion strain, is indeed accumulating in vesicles in the cytosol. About

5 % of the accumulating precursor co-fractionates with mApe1p in the vacuoles (0-19 % fraction).

4.6.2.3 Ficoll-optiprep gradients

In the ficoll floatation gradient (Fig.16b Section 4.6.2.1) and the optiprep sedimentation gradients (Fig.17 Section 4.6.2.2) it was seen that 5 % of the accumulating pApe1p, in the *ΔssaII* cells, co-fractionates with the vacuolar mApe1p. To determine if this pApe1p is present in sub-vacuolar vesicles or in vacuolar vesicles, vacuoles were lysed and separated on an optiprep sedimentation gradient.

ΔssaII cells were spheroplasted using Zymolyase 20 T (3.3.1) and then lysed using water (3.3.2.3). The cell lysate was separated on a ficoll floatation gradient (3.3.7.1). The 0-4 % interface containing vacuoles was collected and separated on an optiprep sedimentation gradient (3.3.7.2). Fractions were collected and TCA precipitated. Proteins were boiled in 1x Laemmli buffer and separated by SDS-PAGE. Western blot analyses were done using antibodies against Ape1p and CPY, which was used as the vacuolar marker.

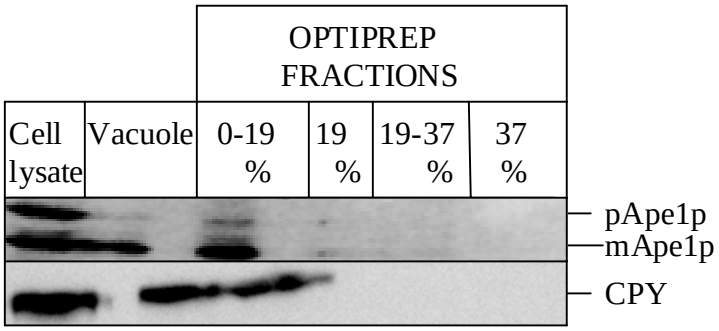


Fig.18: Western blot analyses, of the fractions collected from the vacuoles separated on an optiprep sedimentation gradient, using antibodies against Ape1p and CPY. HRP tagged goat anti-rabbit secondary antibody was used and signals were detected using the Luminol system.

The upper panel in Fig.18 is the western blot analysis done with the Ape1p antibody and the lower panel is the western blot analysis done with the CPY antibody. The first lane is an aliquot of the 'cell lysate' which was used to isolate vacuoles. The second lane is the 'vacuoles' which were isolated and further separated on an optiprep sedimentation gradient. The other lanes are the different fractions collected from the optiprep sedimentation gradient. Cell lysate from the *ΔssaII* cells contains both the accumulating pApe1p and the mApe1p. The vacuoles collected from the 0-4 % interface of the Ficoll gradient (Vacuoles) contain the mature active form of Ape1p (mApe1p) and about 5 % of the accumulating pApe1p. Presence of vacuoles in this fraction is shown by the presence of the vacuolar resident protein CPY in the same fraction (Fig.18, second panel). The accumulating pApe1p co-fractionates with mApe1p in the 0-19 % interface of the optiprep sedimentation gradient. The soluble vacuolar resident protein carboxypeptidase Y (CPY) is also detected in the 0-19 % interface of the gradient (Fig.18, second panel). This indicates that 5 % of the accumulating pApe1p, which co-fractionates with mApe1p, is present in vacuolar vesicles. The pApe1p would have fractionated into the denser regions

of the optiprep gradient if it was present in sub-vacuolar vesicles (Scott *et al*., 1997).

4.7 Presence of Ssa proteins on the vacuolar membrane

In the *ssa* deletion strains we have seen that the pApe1p accumulates as a dodecamer in a vesicle outside the vacuole. There appears to be no defect in the oligomerisation of the protein. So the Ssa proteins might be assisting in the targeting and or fusion of the pApe1p carrying vesicle with the vacuole. In order to perform this function Ssa proteins might be concentrated on the vacuolar membrane. To detect such a concentration of the Ssa proteins, immunoflourescence experiments were done using the *ssa* deletion and the isogenic wild type strain.

WT, *ΔssaI* and *ΔssaI/II* cells were grown to the mid log phase in rich media at 25°C. Cell pellets were fixed and the cell wall was digested using lyticase. Cells were adsorbed to poly-lysine dishes and then incubated with the antibody which identifies both Ssa1p and Ssa2p. Ssa1p and Ssa2p are about 97 % identical and hence the antibody identifies both the proteins. Cy3 conjugated secondary antibody was used. Embedding media supplemented with DAPI was added and covered with the cover slip (3.3.12).

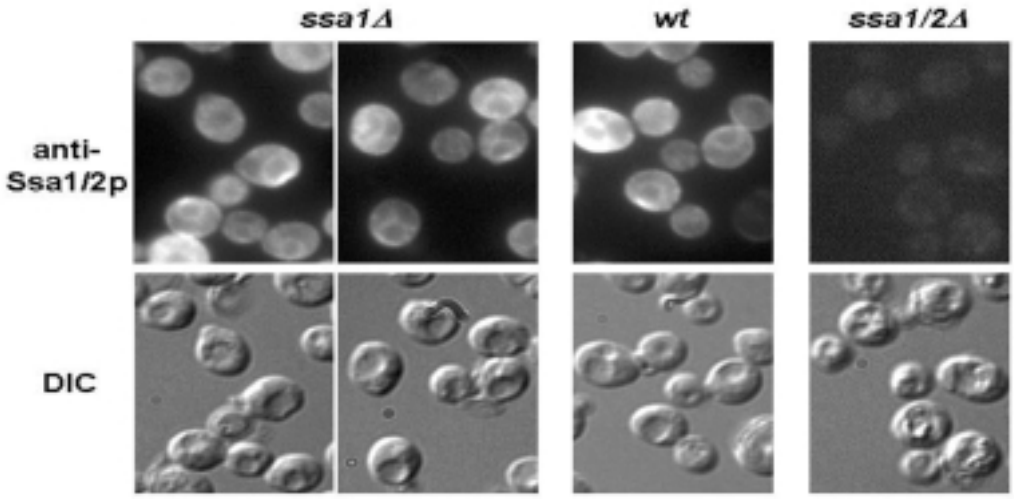


Fig.19: Immunoflourescene microscopy image of the WT, *ΔssaI*, *ΔssaI/II* using an antibody against Ssa1p and Ssa2p. Lower panel is the Nomarski images of the cells.

As shown in Fig.19, in the Wild type (WT) and *ΔssaI* cells cytosolic staining of the Ssa1p and Ssa2p can be seen. The vacuolar membranes in the wild type cells show a prominent labelling of Ssa1p and Ssa2p which exceeds the expected strong cytoplasmic signal. As was seen in case of the WT cells a strong vacuolar membrane staining of Ssa2p is detected in the *ΔssaI* cells. In the *ΔssaI/II* cells no signals could be detected using the Ssa1p and Ssa2p antibody, as was expected.

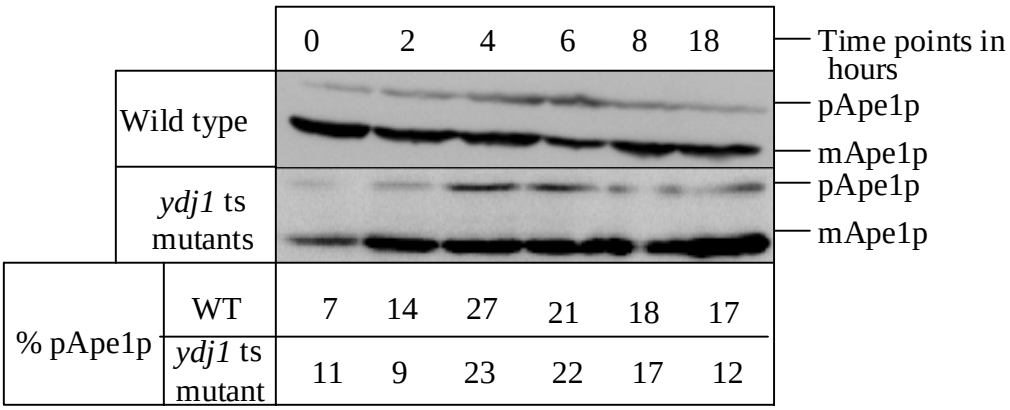
In the lower panel of Fig.19 the Nomarski images of the cells are seen. The vacuoles can be detected in the different cell types as a depression.

4.8 Requirement of Ydj1p for Ape1p transport

Ydj1p is the yeast homolog of the bacterial DnaJ protein. It functions as a cofactor for the HSP70-substrate interaction. Ydj1p interacts with Ssa1p during the transport of proteins into the endoplasmic reticulum and mitochondria (Caplan *et al*., 1992a). *ydj1* temperature sensitive mutants were used to determine if Ydj1p functions as a cofactor along with Ssa proteins in the transport of pApe1p to the vacuole.

4.8.1 Levels of pApe1p and mApe1p in the *ydj1* temperature sensitive mutant at the restrictive temperature

ydj1 ts mutant and the isogenic wild type strain were grown to the mid log phase at the normal growth temperature of 30°C. Cells were then shifted to the restrictive temperature of 37°C. Aliquots were taken 0, 2, 4, 6, 8 and 18 hours after shifting the cells to the restrictive temperature. Cells were spheroplasted using Zymolyase 20T (3.3.1) and lysed using glass beads (3.3.2.1). Proteins were boiled in 1x Laemmli buffer at 95°C for 10 min. Proteins were separated by SDS-PAGE and the western blot analysis was done using an antibody against Ape1p.



% pApe1p	0	2	4	6	8	18
WT	7	14	27	21	18	17
ydj1 ts mutant	11	9	23	22	17	12

Fig.20: Western blot analysis, of the cell extracts from the *ydj1* ts mutants and the isogenic wild type strain, using an antibody against Ape1p. Goat anti-rabbit secondary antibody tagged to HRP was used. Signals were detected using the Luminol detection system.

The upper panel in Fig.20 shows Ape1p, in the cell lysates prepared from the wild type cells collected at different time points at the restrictive temperature. The lower panel shows the cell lysates prepared from the *ydj1* ts mutants after incubation for different time periods at 37°C. As shown in Fig.20, lower panel, there is a slight increase in the amount of pApe1p after incubating the temperature sensitive mutant at 37°C for 4 hours. However, a similar increase in pApe1p is seen in the wild type cells also (Fig.20, upper panel). At the other time points no significant increase in the levels of pApe1p can be seen. This result suggests that-

(1) Ydj1p does not have a role in pApe1p transport or

(2) At the restrictive temperature of 37°C pApe1p takes a Ssa protein independent route into the vacuole.

4.8.2 Levels of pApe1p and mApe1p in *ΔssaI* cells at 37°C

The transport of pApe1p is not affected at the restrictive temperature of 37°C in the *ydj1* ts mutant. To check if pApe1 takes a Ssa protein independent route at 37°C, the levels of pApe1p and mApe1p were detected after *ΔssaI* cells were shifted to the restrictive temperature for various time periods. The transport of pApe1p in the *ΔssaI* cells had been determined under heat shock conditions at 36°C (Fig.3, Section 4.1.2). However, in this experiment the transport phenotype was determined at 37°C (restrictive temperature for the *ydj1* ts mutant).

ΔssaI cells were grown to the mid log phase at 30°C and then shifted to 37°C. Cell aliquots were taken 1, 4 and 18 hours after shifting cells to the higher temperature. Cells were spheroplasted and lysed using glass beads (3.3.2.1). The cell lysates were boiled in Laemmli buffer and separated by SDS-PAGE. Western blot analysis was done using an antibody against Ape1p.

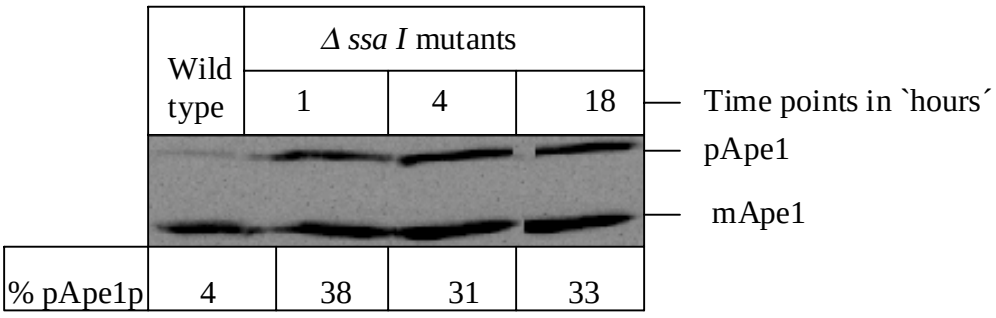


Fig.21: Western blot analysis of the *ΔssaI* cell lysates using an antibody against Ape1p. Goat anti-rabbit secondary antibody tagged to HRP was used. Signals were detected using the Luminol detection system. Cell lysate from the isogenic wild type strain is loaded as a control.

In Fig.21, the first lane is the cell lysate from the wild type strain. Here the mature form of Ape1p (mApe1p) is seen. In the *ΔssaI* cells accumulation of the pApe1p is seen at all the time points (1, 4 and 18 hours) after shifting to 37°C. The accumulation of pApe1p at the restrictive temperature of 37°C shows that Ssa proteins are required for the transport of pApe1p to the vacuole. This result also shows that Ydj1p does not play a role in the cytosol to vacuole targeting of pApe1p.

5. DISCUSSION

In *S. cerevisiae* proteins are transported to the vacuole along four different pathways namely the secretory pathway, Cytosol to Vacuole Targeting pathway (CVT pathway), autophagocytosis and endocytosis. Most of the vacuolar resident proteins, like carboxypeptidase Y and proteinase A, are transported along the secretory pathway (Raymond *et al*., 1992a). These proteins are translocated into the endoplasmic reticulum. In the ER and Golgi they undergo post translational modifications and are then transported into the vacuole along the Vacuolar Protein Sorting (VPS) route (Raymond *et al*., 1992a).

Aminopeptidase 1 (Ape1p) is one of the four leucine aminopeptidases found in *S. cerevisiae* but it is the only one localised to the vacuole (Frey and Röhm, 1978; Matile *et al*., 1971). Ape1p does not have a standard ER targeting signal. The targeting sequence of Ape1p, which is necessary and sufficient to transport the protein into the vacuole, is an amphipathic α-helix and is similar to the mitochondrial targeting signal. In *sec* mutants (mutants in the transport of proteins along the secretory pathway) Ape1p transport is not affected. Over expressed Ape1p accumulates in the cell while over expression of proteins traversing the secretory pathway leads to their secretion. Further, Ape1p has four potential glycosylation sites but the protein is not glycosylated. Based on these findings it was suggested that Ape1p is transported directly from the cytosol into the vacuole along the cytosol to vacuole targeting pathway (cvt pathway) (Klionsky *et al*., 1992). Based on similar observations it has been proposed that the vacuolar inner membrane protein α-mannosidase is also transported along this pathway (Yoshihisa and Anraku, 1990).

Aminopeptidase1 is a homododecameric protein. The oligomeric pApe1p has 12 subunits of 61 kDa each and the mature form of the protein has 12 subunits of 50 kDa each. On transport into the vacuole the 61 kDa precursor (pApe1p) is converted into a 55 kDa intermediate (iApe1p) by proteinase A and

this intermediate is then converted into the 50 kDa mature form (mApe1p) by proteinase B (Seguí-Real *et al*., 1995). This decrease in molecular weight can be used as a marker to detect the transport of the protein into the vacuole.

The aim of this project was to determine, if the cytosolic HSP70s (Heat shock Proteins of the 70 kDa family) belonging to the SSA subfamily assist in the transport of pApe1p to the vacuole.

Heat Shock Proteins of the 70 kDa family (HSP70) are a highly conserved family of proteins known to perform various functions in the cell. They assist in the transport of proteins into organelles, in preventing the misfolding of newly synthesized proteins and in the degradation of the unfolded or wrongly folded proteins.

Cytosol of *S. cerevisiae* contains six heat shock proteins of the 70 kDa family. Two of these, Ssb1 and Ssb2, belong to the SSB subfamily. They are 99% homologous and are known to play a role during protein synthesis (Boorstein *et al*., 1994). About 73 % of the total cellular content of Ssb proteins is associated with the translocating ribosome (Nelson *et al*., 1992). It has been proposed that Ssb proteins bind to the nascent chain as it emerges from the ribosome and this interaction is important for continous and smooth transport of the polypeptide through the ribosomal channel into the cytosol (Nelson *et al*., 1992).

Four other cytosolic members, *SSAI*, *SSAII*, *SSAIII* and *SSAIV*, belong to the SSA subfamily. This is an essential gene family (Werner-Washburne *et al*., 1987). The *SSA1* and *SSAII* genes are constitutively expressed while *SSAIII* and *SSAIV* are expressed under stress conditions (Werner-Washburne *et al*., 1987). The four *SSA* genes have 80-97 % DNA sequence similarity (Craig and Jacobsen, 1985). Strains in which one of the genes has been disrupted do not exhibit any change in the growth phenotype when compared to the wild type cells. The *ssaI/II* double mutant grows slowly at 24°C and does not grow at 37°C. *ssaI ssaII ssaIV* cells are inviable (Deshaies *et al*., 1988). The SSA

subfamily members are known to assist in various functions like assembly of proteins, degradation of wrongly assembled proteins, protein transport etc.

5.1 Ssa proteins in the Cytosol to Vacuole Targeting (CVT) of Aminopeptidase1

In the *ssaI, ssaII* single knock-outs and to a lower extent in the *ssaI/II* double knock-out cells grown at 30°C an accumulation of pApe1p is seen (Fig.2, Section 4.1.1). The pApe1p transport phenotype, seen in the *ssa* deletion cells, is similar to the effect observed on the transport of proteins into the ER and mitochondria. It has been shown that depleting cytosolic HSP70s blocks the import of prepro-α-factor into the ER and the β-subunit of F_1ATPase into mitochondria (Deshaies *et al*., 1988). So the increase in ratio of pApe1p to mApe1p in the *ssa* deletion strains compared to the ratio in the wild type cells indicated that Ssa proteins might play a role in pApe1p transport to the vacuole.

Under conditions of heat stress in the single knock-out strains an accumulation of pApe1p could be seen as was observed when cells were grown at the normal growth temperature. Interestingly however, in the *ssaI/II* double knock-out a wild type phenotype was seen under heat stress conditions (Fig.3, Section 4.1.2). It has been proposed that the Ssa proteins have overlapping functions in the cell and in the absence of one or two of the Ssa proteins the function is taken over by the other members of the subfamily (Werner-Washburne *et al*., 1987). Over expressing *SSAI* under a galactose promoter in a *ssaI ssaII ssaIV* triple knock out could rescue the transport of prepro-α-factor into the ER and the β-subunit of F_1 ATPase into the mitochondria (Deshaies *et al*., 1988). Checking levels of Ssa3/4p in the wild type and *ssa* deletion strains did in fact show a strong over expression of the two stress induced proteins in the double knock-out under heat shock conditions. The levels of Ssa3p and Ssa4p under heat shock conditions in the single knock-out strains, where an

accumulation of pApe1p can be seen, was comparable to the levels in the wild type cells and was insufficient to mediate pApe1p transport (Fig.7, Section 4.3.2). The over expression of Ssa3/4p under heat shock conditions shows that the two stress induced proteins can take over the function, in pApe1p transport, in the absence of Ssa1/2p.

Further, in the double knock-out under normal growth conditions pApe1p accumulation is much less compared to the levels detected in the *ssaI* and *ssaII* single knock-out cells (Fig.2, Section 4.1.1). This is due to the induction of Ssa3/4p expression just by the Ssa1/2p deficiency (Fig.6, Section 4.3.1). Ssa proteins have overlapping functions in the cytosol to vacuole transport of pApe1p and this supports the idea that SSA family members have overlapping functions.

Under heat shock conditions it appears that Ssa3p and Ssa4p take over pApe1p transport into the vacuole completely. However, this reversal of the pApe1p transport phenotype may be an indirect effect of the heat stress. To confirm that over expressed Ssa3/4p can indeed take over pApe1p transport we tried to over express either of these proteins (*SSA3* or *SSA4* under a *GAL* promoter) in the *ssaI* and *ssaII* single knock-out strains. However, in preliminary experiments, the protein profile on SDS-PAGE of the transformant clones was entirely different when compared to that seen in the untransformed cells. HSP70s perform various functions in the cell and hence a massive uncontrolled over expression of these proteins might disrupt many pathways.

In the *ssaII* knock out cells, at 30°C, a low level of expression of Ssa3/4p can be detected (Fig.6, Section 4.3.1). Under normal growth conditions the yeast cytosol contains 30 % Ssa1p and 70 % Ssa2p. Since Ssa2p is the major constitutively expressed Ssa protein, in its absence, Ssa3/4p are expressed under non stress conditions. In the *ΔssaI* cells no Ssa3/4p could be detected. This also supports the view that Ssa proteins not only have overlapping functions, but the total levels of the proteins are responsible for performing a function (Deshaies *et al*., 1988).

The wild type phenotype exhibited by the *ΔssaII* mutants, on expressing *SSAII* under its endogenous promoter in a single copy plasmid, confirmed the involvement of HSP70s in pApe1p transport (Fig.4, Section 4.2.1).

Ssa proteins are known to assist in the transport of proteins into various organelles. In most of the transport processes one of the two constitutively expressed Ssa proteins has been shown to be more involved than its homologue.

(1) Ssa1p is known to assist in the translocation of proteins into the ER and mitochondria *in vivo* (Deshaies *et al*., 1988).

(2) Ssa2p is involved in the vesicle mediated transport of fructose 1-6 bisphosphatase (FBPase) to the vacuole (Brown *et al*., 2000). FBPase is an enzyme involved in gluconeogenesis. If cells are shifted from gluconeogenic media to glucose containing media FBPase will be degraded. One of the mechanisms proposed for the degradation of this enzyme is a vesicle mediated process.

(3) Recently using an affinity column Silles *et al*, have shown that Ssa1p binds to the presequence of pApe1p. The authors have interpreted their data in a way that Ssa1p targets pApe1p to the cvt vesicle membrane.

In order to show such a direct interaction between pApe1p and the Ssa proteins we tried to chemically cross link the two proteins *in vivo*. The cross linked products were immunoprecipitated with antibodies against Ssa1p and Ssa2p and immunostained using the Ape1p antibody. However, we were not able to detect significant amounts of cross linked product. This could be because Ssa proteins are known to interact with many proteins in the cytosol and hence the detection, of the cross linked and immunoprecipitated proteins, with the Ape1p antibody might not be very sensitive. So the next approach was using a hexahistidine tagged Ape1p. Cells were transformed with a plasmid containing pApe1p with a C-terminal hexahistidine tag and under the control of the alcohol dehydrogenase (ADH) promoter. The idea was to bind the cross linked proteins to the nickel agarose column and then elute them using

antibodies against Ssa1/2p. However, on over expression, the hexahistidine tagged pApe1p was degraded. Hence, we were not able to show a direct interaction between pApe1p and the cytosolic Ssa proteins.

We detect an accumulation of pApe1p in both *ssaI* and *ssaII* single knock-out cells (Fig.2, Section 4.1.1), indicating that both Ssa1p and Ssa2p play a role in the cytosol to vacuole targeting of pApe1p. A phenotype detected in both the cell types raises the possibility that Ssa1p and Ssa2p might be playing a role at two unique steps in the transport process. However, a wild type phenotype seen when Ssa1p was over expressed in the *ΔssaII* cells and vice versa showed that the Ssa1p and Ssa2p perform overlapping functions in the cell (Fig. 5, Section 4.2.2).

5.2 Requirement of Ssa proteins for autophagocytosis

Autophagocytosis is a pathway by which cytosolic proteins and organelles are transported to the vacuole for degradation under starvation conditions (Dunn, 1994; Mortimore *et al*., 1989; Schworer *et al*., 1981). Most of the data suggest that the mechanism by which proteins are transported to the vacuole during autophagy and by the cvt pathway are similar (Baba *et al*., 1997; Scott *et al*., 1997). In both cases a double membrane vesicle is formed in the cytosol which engulfs the cargo and transports it to the vacuole. The outer membrane of the vesicle (autophagosome, cvt vesicle) fuses with the vacuolar membrane releasing a single membraned vesicle (autophagic body, cvt body) into the vacuole. The contents are subsequently released into the vacuole. However, the two pathways differ in that-

(i) The cvt pathway occurs constitutively in the cell and is a biosynthetic route. Autophagy occurs under nutrient starvation conditions and is a degradative process.

(ii) The cvt vesicle is about 150 nm in diameter while the autophagosome is about 300-900 nm.

It has been shown that many of the proteins required for autophagy are also required for the cvt pathway. Many of the genes identified by genetic screening proceedures are shown to be involved in both pathways-

PROTEIN	**TOPOLOGY/ LOCALISATION**	**FUNCTION/ INTERACTION**
Apg1/ Aut3/ Cvt10	Hydrophilic, cytosolic.	Ser/Thr protein kinase,over expression suppresses *apg13* (Funakoshi *et al.*, 1997). Apg1p is not required for phosphorylation of Apg13p or Vac8p (Scott *et al*., 2000)
Apg5	Hydrophilic, membrane associated.	Involved in the sequestration step and required for vesicle formation and/ or completion. Conjugated by Apg12p and binds Apg16p (George *et al.*, 2000).
Apg7/ Cvt2	Soluble, punctate staining in SD-N.	Homologous to ubiquitin-activating enzymes (E1), required for Apg12p-Apg5p conjugation. Required for vesicle formation (Kim *et al*., 1999).
Apg9/ Cvt7	Integral membrane Protein	Required for vesicle formation. (Noda *et al.,* 2000)
Apg10	-----	Required for Apg12p-Apg5p conjugation. Required for vesicle formation (Mizushima *et al*., 1998).
Apg12	Hydrophilic, Membrane associated.	Conjugated to Apg5p, phosphorylated. Required for vesicle formation. (Mizushima *et al.*, 1998)
Apg13p and Vac8p	Membrane associated	Are part of a larger complex of phosphorylated proteins, which might include Apg1p. Required for vesicle formation (Scott *et al*., 2000)
Apg16	Hydrophilic	Binds Apg5p. Cross links Apg5p-Apg12p conjugates. Required for vesicle formation/ completion. (Mizushima *et al*., 1999)

Aut2/ Apg4	Cytosolic	Interacts with Tub1p, Tub2p and Aut7p to move vesicle to vacuole (Lang *et al*., 1998).
Aut7/ Apg8/ Cvt5	Cytosolic	Homologue of rat microtubule associated LC3, interacts with Aut2p. (Lang *et al*., 1998). Required for autophagosome/ CVT vesicle formation (Kirisako *et al*., 1999).

Table 1: Modified from Klionsky and Ohsumi, 1999.

However, in *aut4* and *aut6* mutants the transport of proteins to the vacuole by autophagy is disrupted while the cvt pathway is not affected (Harding *et al*., 1996). Tlg2p (t-SNARE of the late Golgi) is localised to the late Golgi and endosomes. It is required for the cvt pathway while autophagy is not affected in the *Δtlg2* cells (Abeliovich *et al*., 1999).

Similarly, in the *ssa* deletion strains we found that the transport of Ape1p to the vacuole was normal under autophagy conditions (Fig.8, Section 4.4). As mentioned previously an over expression of Ssa3/4p, under heat shock conditions, can take over the transport of pApe1p completely (Fig.7, Section 4.3.2; Fig.3, Section 4.1.2). Checking the levels of Ssa3/4p, in the *ΔssaII* cells, under autophagy conditions we found that the expression was less than that seen in the *ΔssaII* cells grown at 30°C in nutrient rich media (YPD) (Fig.10, Section 4.4). When grown at 30°C in YPD *ΔssaII* cells show an accumulation of pApe1p, however, no accumulation is seen under autophagy conditions. Similarly, the Ssa3/4p expression level in the *ΔssaII* cells grown to stationary phase in YPD media is lower than the expression in the *ΔssaI/II* at 30°C, where Ssa3/4p levels were insufficient to restore the pApe1p transport completely. From this we conclude that Ssa proteins are only involved in the cvt pathway but not in the autophagic pathway. There is however the possibility that pApe1p transport by autophagy has lower Ssa protein requirement than the cvt

pathway. Autophagosomes are about 6-12 times bigger than cvt vesicles so an autophagy mediated transport event could yield upto 6-12 times more pApe1p in the vacuole compared to a single cvt vesicle mediated transport event.

As mentioned previously autophagocytosis involves a non-specific uptake of cytosolic components and organelles. An autophagosome is not entirely filled with pApe1p but contains other cargo as well, as shown by electron microsocopy (Baba *et al*., 1997). Infact electron microscopy images, presented by Baba *et al* in 1997, show that an autophagosome does not contain more pApe1p than a cvt vesicle. Moreover, Ssa proteins are required for different cellular processes and their functions become more essential under heat shock conditions resulting in an induction of Ssa protein expression. The low expression levels of Ssa proteins in the *ΔssaII* cells under autophagy conditions makes it more unlikely that they are able to mediate efficient pApe1p transport. This indicates that the transport of pApe1p by cvt vesicles and autophagosomes might have different Ssa protein requirements.

5.3 Role of Ydj1 co-chaperone in the cytosol to vacuole targeting of pApe1p

The interaction of HSP70s with their protein substrates is tightly coupled to their ATPase cycle (Bukau and Horwich, 1998). The ATPase activity of HSP70s is extremely weak and hence co-chaperones such as DnaJ (in *E. coli*) are needed for stimulation of ATP hydrolysis (McCarty *et al*., 1995; Szabo *et al*., 1994; Liberek *et al*., 1991), while others facilitate (GrpE and BAG-1) (Höhfeld and Jentsch, 1997; McCarty *et al*., 1995; Szabo *et al*., 1994; Liberek *et al*., 1991) or even prevent (HIP) nucleotide release (Höhfeld *et al*., 1995). Ydj1p, the yeast homolog of the *E. coli* DnaJ protein, is shown to be a cytosolic protein that is partially membrane bound via modification with a farnesyl lipid moiety at its C-terminus (Caplan *et al*., 1992b; Caplan and

Douglas, 1991). Ydj1p can stimulate the ATPase activity of HSP70, leading to dissociation of HSP70-polypeptide complexes (Cyr *et al*., 1992). It has been shown that purified Ydj1p, which has no ATPase activity itself, can stimulate the ATPase activity of Ssa1p approximately 9 fold, as well as catalyse the dissociation of Ssa1-polypeptide complexes (Cyr *et al*., 1992). Ydj1p is required for the efficient import of $F_1\alpha$, $F_1\beta$ and $F_1\gamma$ proteins into the mitochondria and prepro-α-factor into the ER (Caplan *et al*., 1992a).

We have seen that HSP70s are required for the transport of pApe1p to the vacuole along the cvt pathway. So we wanted to determine if Ydj1p functions as a co-factor for HSP70 in this process. Using a *ydj1* temperature sensitive mutant we found that there was no block in the transport of pApe1p into the vacuole (Fig.20, Section 4.8.1). However, at the restrictive temperature of 37°C pApe1p could take a Ssa protein independent route in the *ydj1* ts mutant, as seen previously under autophagy conditions, where pApe1p takes an Ssa protein independent route into the vacuole (Fig.8 and Fig.9, Section 4.4). When the *ΔssaI* cells were shifted to the restrictive temperature, accumulation of pApe1p was still observed (Fig.21, Section 4.8.2). This shows that pApe1p transport into the vacuole is a Ssa protein dependent, Ydj1p independent pathway.

It has been shown previously that Ydj1p is not required for all the Ssa protein dependent transport pathways. Translocation into the ER of the soluble vacuolar resident protein CPY, vacuolar membrane protein DPAP-B and the ER resident protein BiP is dependent on Ssa1p (Deshaies *et al*., 1988) while no transport defect was seen in the *ydj1* ts mutants (Caplan et al., 1992a). Similarly in *E. coli* DnaK is known to prevent the heat induced denaturation of RNA polymerase. It also reactivates the heat inactivated RNA polymerase by dissolving the aggregates. Both these reactions are known to occur in the absence of DnaJ *in vitro* (Skowrya *et al*., 1990).

Certain amino acids have the ability to increase the ATPase activity of DnaK in the absence of DnaJ. *In vitro* experiments have shown that isoleucine

enhances the ATPase activity of DnaK four fold in the absence of DnaJ and there is no further increase in the ATPase activity when DnaJ is added. Phenylalanine, leucine and valine also show a similar effect (Richarme and Kohiyama, 1993). Arginine and lysine are known to enhance ATPase activity by two fold, however, their ability to enhance ATPase activity increases in the presence of DnaJ.

5.4 Oligomeric status of the accumulating pApe1p in the *ssa* deletion strains

Ape1p is a dodecameric protein. The transport of pApe1p from the cytosol to the vacuole occurs post translationally (Seguí-Real *et al*., 1995; Klionsky *et al*., 1992).

Seguí-Real *et al*., suggest that the oligomerisation of Ape1p occurs in the vacuole. They used a strain containing pApe1p with a 'myc' tag at its C-terminus to show that the transport of pApe1p is a translocation process. The construct was found stuck in a membrane and the N-terminus was cleaved giving rise to the intermediate form of the protein. This conversion of pApe1p to the intermediate form is dependent on proteinase A which is found in the vacuole. Based on these observations they suggested that pApe1p subunits enter the vacuole by translocation and then oligomerisation and maturation occur. This mechanism would be similar to the transport of proteins into the ER and mitochondria, where proteins enter the organelles in an extended conformation and fold into the functional conformation on entering the organelle (Schatz and Dobberstein, 1996). Cytosolic Ssa proteins are known to assist in retaining the proteins, destined to the ER and mitochondria, in a translocation competent conformation (Schatz and Dobberstein, 1996). The Ssa proteins might be performing an identical function by maintaining the pApe1p subunit in a translocation competent state.

Kim *et al*., suggest that pApe1p enters the vacuole as a fully assembled dodecamer. The transport of an assembled dodecamer into the vacuole is reminiscent of the transport of fully assembled oligomers into peroxisomes. 3-keto acyl CoA thiolase enters the peroxisomes as a dimer (Glover *et al*., 1994), while a chimera of chloramphenicol acetyl transferase with PTS1 (Peroxisomal targeting signal 1) is transported to the peroxisomes as a trimer (McNew and Goodman, 1994). Pulse chase analysis suggested that the oligomerisation of pApe1p is an early step in the transport process. The half time of transport of pApe1p to the vacuole is approximately 45 min while the oligomerisation occurs within 2 min. So it has been proposed that pApe1p oligomerisation occurs in the cytosol and then it binds to a membrane (Kim *et al*., 1997).

When proteins from the *ssaII* deletion strain were separated on glycerol density gradients, based on their molecular weight, the accumulating pApe1p and mApe1p co-fractionated along with the 669 kDa thyroglobulin marker (Fig.11, Section 4.5.1). This suggested that the accumulating pApe1p in the *ssa* deletion strains is assembled into the 732 kDa dodecamer.

Though the accumulating pApe1p is assembled into a dodecamer, in the *ssaII* deletion strain, the possiblity of a misassembly of the protein could not be ruled out. The wrongly assembled protein could be degraded in the vacuole in a proteinase A dependent manner leading to a decrease in the total levels of Ape1p in the *ssa* deletion strains. It is interesting to note that a decrease in the total levels of Ape1p was observed in the *ΔssaI* and *ΔssaII* cells, grown at 30°C, when compared to the levels of the protein in the isogenic wild type cells (Fig.2, Section, 4.1.1). However, similar levels of Ape1p detected in cells lacking *ssaI* and cells lacking both *ssaI* and *pep4* (*PEP4* encoding for proteinase A, required for the activation of other vacuolar proteases), indicated that the assembly of the dodecamer is not affected (Fig.13, Section 4.5.3). This was further supported by the similar specific enzymatic activity of the vacuolar mApe1p in the wild type and *ΔssaII* strains. An increase in the specific activity was observed in the *ΔssaI* and *ΔssaI/II* cells (Fig.14, Section 4.5.4). The

specific vacuolar activity detected in the *ΔssaI/II* cells not only indicates that the pApe1p is correctly assembled into a dodecamer, but it also shows that the Ssa3/4p are taking over the function of Ssa1/2p, thus confirming that the four Ssa proteins are playing a role at the same step in the transport process.

These results showed that a decrease in the levels of cytosolic HSP70s leads to an accumulation of pApe1p which is already assembled into a dodecamer.

5.5 Localisation of the accumulating pApe1p in the *ssa* deletion strains

In the *Δssa* strains pApe1p is accumulating as a fully assembled dodecamer. This led to the next question as to where the oligomer is accumulating in the cell, cytosol, cvt vesicle or vacuole.

Analysis of the CVT mutants (Harding *et al*., 1996) indicated that there is a substantial overlap between the genes involved in Ape1p transport and autophagocytosis (Refer Table:1). In addition, phenotypic analysis of cvt mutants and autophagy mutants showed that most of these mutants are defective in autophagy and also accumulate pApe1p. Further, the fact that pApe1p transport is sensitive to low temperatures and requires a GTP binding protein *in vitro* (Scott *et al.*, 1996) indicated that it is a vesicle mediated process. Immunoelectronmicroscopy using *ape1* targeting mutants showed pApe1p in vesicles in the cytosol. Similarly immunoelectronmicroscopy done with autophagy mutants showed pApe1p in vesicles accumulating inside vacuoles. Based on these observations it was suggested that pApe1p transport to the vacuole is a vesicle mediated process. If the transport of the dodecameric pApe1p to the vacuole is a vesicle mediated process, then the cytosolic HSP70s might be assisting in the binding of the dodecamer to the membrane, in the formation/ completion of the vesicle or in the targeting and/ fusion of the vesicle with the vacuole.

Membrane protection studies on the accumulating pApe1p, seen in the *ssa* deletion strains, showed that the protein was accessible to proteinase K only in the presence of a detergent (Fig.15, Section 4.6.1). This suggests that the accumulating pApe1p is membrane protected. However, another explanation of this result could be that the precursor is associated with a membrane in such an orientation that it remains inaccessible to ProK in the absence of a detergent. Due to the complete disruption of the membrane by detergent (tritonX-100) pApe1p is digested by proteinase K. However, using mild techniques to disrupt membranes, like freeze thaw and freeze thaw sonication, we still found that the accumulating pApe1p is membrane protected and not just membrane associated.

Further, density gradient centrifugation studies showed that the accumulating pApe1p is not present in the vacuoles. In the gradients the accumulating pApe1p fractionated into the more dense regions or into the pellet of the gradient (Fig.16b, section 4.6.2.1; Fig.17, Section 4.6.2.2). Similarly, in the *vps18* (VPS: Vacuolar Protein Sorting) mutant the accumulating pApe1p was found to accumulate in a non-vacuolar vesicular compartment (Scott *et al*., 1997). Vps18p is known to play a role in the late stage of vacuolar protein sorting (Preston *et al*., 1991; Robinson *et al*., 1991). However, in the *ape1* mutant, where proline in the targeting signal is replaced by leucine (P22L), the precursor was found in the pellet fraction of the ficoll gradient but it was accessible to proteinase K even in the absence of a detergent. This showed that accumulating pApe1p, in the P22L mutant, is membrane associated (Scott *et al*., 1997). However, in the *ssaII* deletion strain we see that the accumulating pApe1p is membrane protected and not co-fractionating with the vacuoles. A small amount of pApe1p (about 5 % of the total accumulating pApe1p) which co-fractionated with the vacuoles was found to be associated with the vacuoles and not in sub-vacuolar vesicles (Fig.18, Section 4.6.2.3), indicating that the pApe1p is accumulating in vesicles outside the vacuole.

It has been suggested that large protein complexes are required for autophagocytosis as well as cytosol to vacuole targeting of pApe1p. Formation of these complexes are required for autophagy not only in yeast but in mammals also (Mizushima *et al*., 1998). Apg12p and Apg5p are both membrane associated proteins. The carboxy terminal glycine residue of Apg12p is conjugated to lysine on Apg5p thus forming an ubiquitination like conjugation (Mizushima *et al*., 1998). This conjugation is assisted by Apg7p/ Cvt2p and Apg10p. Apg7p, homologous to the ubiquitin activating enzyme E1, activates Apg12p while Apg10p might be an E2 (ubiquitin conjugating enzyme) like protein (Mizushima *et al*., 1998). The Apg12p/Apg5p conjugate then interacts with Apg16p to form a multimeric complex (Mizushima *et al*., 1999). This conjugation is shown to be required for both autophagy, under starvation conditions, and cvt pathway, under constitutive growth conditions. It is proposed that this conjugation is required for vesicle (autophagosome, cvt vesicle) formation or completion (George *et al*., 2000; Kim *et al*., 1999; Mizushima *et al*., 1999).

However, our results show that in the *ΔssaII* cells pApe1p is accumulating as a dodecamer in a membrane bound compartment outside the vacuole. These observations rule out a possible role for HSP70s in the oligomerisation of pApe1, or in the formation/ completion of the vesicle.

5.6 Role of cytosolic HSP70s in the cytosol to vacuole targeting of pApe1p

A decrease in the levels of Ssa proteins leads to an accumulation of the pApe1p. The accumulating protein is correctly assembled into a dodecamer and is localised in vesicles in the cytosol. These results support the proposition that Ssa proteins might be assisting in the targeting and or fusion of the vesicle containing pApe1p with the vacuole.

If the Ssa proteins are assisting in the targeting/ fusion of the cvt vesicle with the vacuole they could be concentrated on the vacuolar membrane. The constitutively expressed Ssa1/2p could be detected on the vacuolar membrane by immunofluorescence using the Ssa1/2p antibody (Fig.19, Section 4.7). However, the stress induced Ssa3/4p could not be detected. Western blot analysis of vacuoles isolated from the wild type cells with the anti-Ssa1/2p antibody detected HSP70s associated with vacuoles. Further efforts to localise the Ssa proteins to the outer or inner surface of the vacuolar membrane by immunoelectron microscopy did not yield definitive results.

It would be interesting to determine if HSP70s are present within the vacuole in *S. cerevisiae*. HSP70s have been found in the lumen of the ER and mitochondria and are known to assist in the transport and folding of the incoming proteins into their functional conformation (Schatz and Dobberstein, 1996). HSC73 is detected in purified lysosomes, as shown by Agarraberes *et al*., in 1997.

The localisation of Ssa1/2p on the vacuolar membrane further supports a function for these proteins in the fusion of the cvt vesicle with the vacuole.

Membrane fusion events have been extensively studied in *S. cerevisiae* as well as neurons. Similar proteins are known to catalyse the event in both the systems thus establishing their generality (Scheckman, R. 1998; Bock, J.B and Scheller, R.H. 1997). Vesicles in many trafficking reactions utilise homologous GTPases, N-ethylmaleimide Sensitive Factor (NSF), SNARE (Soluble NSF Attachment Protein Receptor), SNAP (Soluble NSF Attachment Protein) and other proteins to catalyse ‘docking’ to the target membrane followed by fusion (Weis, W.I. and Scheller, R.H. 1998; Rothman, J.E. and Wieland, F. 1996; Ferro-Novick, S. and Jahn, R. 1994; and Rothman, J.E. 1994).

In *S. cerevisiae*, isolated vacuoles bear a multisubunit ‘cis-SNARE complex’ consisting of Vam3p (SNARE on the target membrane, t-SNARE), Vam7p (SNAP 23/25 homolog), Nyv1p/ Vti1p/ Ykt6p (SNAREs on the vesicle membrane, V-SNARE), LMA1 (Low Molecular Activity 1) which is a

heterodimeric protein (Ungermann *et al*., 1999a; Ungermann *et al*., 1999b; Ungermann and Wickner, 1998; Ungermann *et al*., 1998; Nichols *et al*., 1997). In addition to this Sec18p (NSF) ATPase, a chaperone, and its cochaperone Sec17p (α-SNAP) are part of this complex (Ungermann *et al*., 1998; Mayer *et al*., 1996).

The over all reaction occurs in three stages: priming, docking and fusion.

(1) Priming: ATP hydrolysis by Sec18p leads to the release of Sec17p from the vacuoles. This is followed by the disassembly of the cis-SNARE complex. The t-SNARE is activated and LMA1, which is associated with Sec18p, is transfered to the t-SNARE which it stabilizes. (Ungerman *et al*., 1999a)

(2) Docking: The reversible association of the vacuoles, catalysed by a Rab like protein Ypt7p, is termed 'tethering' (Ungermann *et al*., 1998; Mayer and Wickner, 1997).

A large multisubunit protein called the Vam2/6p complex is bound to the cis paired SNAREs. This interaction is disrupted during priming. The Vam2/6p then binds to Ypt7p to initiate productive contact between the vacuoles (Price *et al*., 2000). Since Vam2/6p is required on both the partner vacuoles (Price *et al*., 2000) the two complexes, on apposing vacuoles may contact during docking. Trans-SNARE pair formation stabilizes the association between tethered vacuoles, to complete docking. It has been suggested that Vam2/6p might promote trans-SNARE pairing (Price *et al*., 2000).

(3) Fusion: The fusion of docked vacuoles does not require ATP, Sec17p or Sec18p or continued presence of the trans-SNARE pairs (Ungermann *et al*., 1998; Mayer *et al*., 1996; Conradt *et al*., 1994). Vacuole fusion requires an efflux of calcium from the vacuolar lumen (Peters and Mayer, 1998). Release of calcium into the cytosol results in transient association of calmodulin (cytosolic calcium binding protein), which triggers the final events of bilayer and contents mixing (Peters *et al*., 1999). LMA1

release is the last biochemically defined event that preceeds fusion (Xu *et al*., 1998).

In the *Δssa* mutants we see an accumulation of pApe1p in vesicles outside the vacuole. The mechanism of cvt vesicle to vacuole fusion is not known. It has been suggested that chaperones support the formation of NSF, SNARE complexes on the membranes required for docking and fusion (Ungermann *et al*., 1999b). So Ssa1/2p might be required for the heterotypic fusion between the cvt vesicle and vacuole. Vam3p is a vacuolar t-SNARE required for the fusion of vesicles from the cvt, secretory and autophagy pathways (Darsow *et al*., 1997) and also in the homotypic vacuole fusion (Nichols *et al*., 1997). So the cvt pathway may be using the same mechanisms required for homotypic vacuole fusion. However, it is important to note that in *Δssa* strains no defect is detected in the transport of CPY to the vacuole.

We attempted to set up an *in vitro* fusion assay to determine if the cytosolic HSP70s are required for heterotypic vesicle to vacuole fusion. We also wanted to determine if the cytosolic or membrane associated HSP70s or both are required for the fusion reaction. The fusion assay was performed using permeabilised wild type and *ΔssaII* cells. Cytosol containing normal levels (from wild type cells) or decreased levels (from *ΔssaII* cells) of Ssa proteins was added and the reaction was done in the presence or absence of ATP. The fusion event could be detected by a decrease in the levels of pApe1p and a simultaneous increase in the levels of mApe1p, as seen by western blot analysis with Ape1p antibody. The other marker to indicate transport would have been a shift in pApe1p from the pellet, containing cvt vesicles, to the 0-4 % interface of the ficoll gradient, containing vacuoles, after the cells had been incubated together in the presence of sufficient levels of Ssa proteins. However, the assay was not sensitive enough to detect either of these changes.

In the future other approaches would have to be used to set up an *in vitro* assay for the heterotypic fusion reaction. This would not only help to determine a novel function for HSP70s in vesicle to vacuole fusion, but would also help in

detecting other factors involved in this heterotypic fusion process. The cytosol to vacuole targeting of pApe1p provides a good system since the decrease in molecular weight of the protein, on entering the vacuole, serves as a useful marker for detecting the fusion reaction.

6. SUMMARY

In *Saccharomyces cerevisiae*, aminopeptidase 1 (Ape1p) is a vacuolar resident protein which is transported to the vacuole by a non classical pathway. On synthesis in the cytoplasm the protein assembles into a homododecamer and is transported directly into the vacuole. It does not take the classical secretory pathway which is taken by most of the vacuolar resident proteins. A translocation as well as a vesicle mediated process have been suggested to describe the cytosol to vacuole targeting of Ape1p.

In this project we determined that the cytosolic members of the heat shock proteins of the 70 kDa family (HSP70s) assist in the transport of aminopeptidase 1 to the vacuole (Satyanarayana *et al*., 2000). In *S. cerevisiae* HSP70 members belonging to the SSA subfamily are known to assist in protein targeting to the ER and mitochondria. Using strains lacking either one *(ΔssaI*, *ΔssaII*) or both *(ΔssaI/II*) the constitutively expressed Ssa proteins we found that the precursor form of Ape1p (61 kDa on an SDS polyacrylamide gel) accumulates. However, in the isogenic wild type cells only the mature form of Ape1p could be detected (50 kDa on an SDS polyacrylamide gel) indicating normal transport. In the absence of the constitutively expressed Ssa1/2p the stress induced Ssa3/4p could take over the transport of pApe1p. This showed that the Ssa proteins perform overlapping functions.

The accumulating precursor Ape1p in the *Δssa* strains was found to accumulate as a dodecamer and there appeared to be no misassembly of the protein. This ruled out the possibility that the cytosolic HSP70s might be assisting in the oligomerisation of the protein. Further, we found that the accumulating pApe1p was membrane protected and density gradient centrifugation showed that the protein was not accumulating in the vacuole. These results together showed that a decrease in the levels of cytosolic HSP70s leads to the accumulation of pApe1p as a dodecamer in vesicles in the cytosol. Detection of Ssa proteins on the vacuolar membrane, using

immunofluorescence microscopy, suggested that the HSP70s might be assisting in the fusion of the cvt vesicle with the vacuole. Further efforts to determine if the cytosolic HSP70s are indeed assisting in heterotypic vacuole fusion (cvt vesicle to vacuole), using an *in vitro* fusion assay, did not yield definitive results.

The vesicle mediated transport of Ape1p to the vacuole is suggested to be mechanistically similar to the non-specific uptake of proteins and organelles into the vacuole by autophagy. Many of the factors are required for both the cvt pathway, which occurs constitutively in the cell, and autophagy which occurs under nutrient starvation conditions (Refer Table 1, Section 5.2). However, we found that in the *ssa* mutants the transport of Ape1p to the vacuole occurs normally under starvation conditions, while an accumulation of the precursor form is seen under normal growth conditions. These results showed that cytosolic HSP70s are required for the cytosol to vacuole transport of Ape1p and not for the autophagy mediated transport of the protein.

The interaction between the HSP70s and the substrates is an ADP/ATP cycle. In the ADP bound form of HSP70 the substrate binds tightly to the chaperone while exchange of ADP for ATP releases the substrate. DnaJ (in *E. coli*) is known to enhance the ATPase activity of DnaK (*E. coli* HSP70) while GrpE (in *E. coli*) assists in the ADP-ATP exchange. Ydj1p is a DnaJ homolog found in the cytosol of *Saccharomyces cerevisiae*. It is known to assist the cytosolic HSP70, Ssa1p, in the translocation of proteins into the ER and mitochondria. However, using *ydj1* temperature sensitive mutants we found that the transport of Ape1p to the vacuole was not affected. This showed that the transport of Ape1p to the vacuole is a Ssa protein dependent and Ydj1p independent process.

7. REFERENCES

Abeliovich, H., Darsow, T. and Emr, S.D. (1999). Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. *EMBO J*. **18**, 6005-6016.

Agarraberes, F.A., Terlecky, S.R. and Dice, J.F. (1997). An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J. Cell Biol.* **137**, 825-834.

Ahlberg, J. and Glaumann, H. (1985). Uptake-microautophagy-and degradation of exogenous proteins by isolated rat liver lysosomes. Effects of pH, ATP, and inhibitors of proteolysis. *Exp. Mol. Pathol*. **42**, 78-88.

Ammerer, G., Hunter, C.P., Rothman, J.H., Saari, G.C., Valls, L.A. and Stevens, T.H. (1986). *PEP4* gene of *Saccharomyces cerevisiae* encodes Proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. And Cell. Biol.* **6**, 2490-2499.

Anraku, Y., Umemoto, N., Hirata, R. and Wada, Y. (1989). Structure and function of the yeast vacuolar membrane proton ATPase. J. *Bioenerg. Biomembr*. **21**, 589-603.

Atencio, D.P. and Yaffe, M.P. (1992). *MAS5*, a yeast homolog of DnaJ involved in mitochondrial import. *Mol. Cell Biol*. **12**, 283-291.

Azem, A., Oppliger, W., Lustig, A., Jenö, P., Feifel, B., Schatz, G. and Horst, M. (1997). The mitochondrial hsp70 chaperone system. Effect of adenine nucleotides, peptide substrate, and mGrpE on the oligomeric state of mhsp70. *J. Biol. Chem*. **272**, 20901-20906.

Baba, M., Osumi, M., Scott, S.V., Klionsky, D.J. and Ohsumi, Y. (1997). Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/ lysosome. *J. Cell Biol.* **139**, 1687-1695.

Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994). Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterisation. *J. Cell Biol*. **124**, 903-913.

Banta, L.M., Robinson, J.S., Klionsky, D.J. and Emr, S.D. (1988). Organelle assembly in yeast: Characterisation of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol*. **107**, 1369-1383.

Beckmann, R.P., Mizzen, L.E. and Welch, W.J. (1990). Interaction of hsp70 with newly synthesized proteins: implications for protein folding and assembly. *Science* **248**, 850-854.

Blond-Elguindi, S., Cwirla, S.E., Dower, W.J., Lipshutz, R.J., Sprang, S.R., Sambrook, J.F. and Gething, M.J. (1993). Affiniy panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* **75**, 717-728.

Bock J.B. and Scheller, R.H. (1997). Protein transport. A fusion of new ideas. *Nature* **387**, 133-135.

Bolliger, L., Deloche, O., Glick, B.S., Georgopoulos, C., Jenö, P., Kronidou, N.G., Horst, M., Morishima, N. and Schatz, G. (1994). A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability. *EMBO J*. **13**, 1998-2006.

Boorstein, W.R., Ziegelhoffer, T. and Craig, E.A. (1994). Molecular evolution of the HSP70 gene family. *J. Mol. Evol.* **38**, 1-17.

Braulke, T., Gartung, C., Hasilik, A. and von Figura, K. (1987). Is movement of mannose-6-phosphate specific receptor triggered by binding of lysosomal enzymes? *J. Cell Biol.* **104**, 1735-1742.

Brown, C.R., McCann, J.A. and Chiang, H.L. (2000). The heat shock protein Ssa2p is required for import of fructose-1,6-bisphosphatase into Vid vesicles. *J. Cell Biol*. **150**, 65-76.

Buchberger, A., Theyssen, H., Schröder, H., McCarty, J.S., Virgallita, G., Milkereit, P., Reinstein, J. and Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J. Biol. Chem*. **270**, 16903-16910.

Bukau, B. and Horwich, A.L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351-366.

Caplan, A.J., Cyr, D.M. and Douglas, M.G. (1992a). Ydj1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. *Cell* **71**, 1143-1155.

Caplan, A.J., Tsai, J., Casey, P.J. and Douglas, M.G. (1992b). Farnesylation of Ydj1p is required for function at elevated growth temperatures in *S.cerevisiae*. *J. Biol Chem.* **267**, 18890-18895.

Caplan, A.J. and Douglas, M.G. (1991). Characterization of YDJ1: a yeast homologue of the *E.coli DNAJ* gene. *J. Cell Biol.* **114**, 609-622.

Chang, Y.H. and Smith, J.A. (1989). Molecular cloning and sequencing of genomic DNA encoding Aminopeptidase I from *Saccharomyces cerevisiae. J. Biol. Chem*. **264**, 6979-6983.

Chappel, T.G., Welch, W.J., Sclossman, D.M., Palter, K.B., Schlesinger, M.J. and Rothman, J.E. (1986). Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* **45**, 3-13.

Chiang, H.L., Terlecky, S.R., Plant, C.P. and Dice, J.F. (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246**, 382-385.

Chiang, H.L. and Dice, J.F. (1988). Peptide sequences that target proteins for enhanced degradation during serum withdrawal. *J. Biol. Chem*. **263**, 6797-6805.

Chirico, W.J., Walters, M.G. and Blobel, G. (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332**, 805-810.

Chvatchko, Y., Howald, I. and Riezman, H. (1986). Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell* **46**, 355-364.

Conibear, E. and Stevens, T.H. (1995). Vacuolar biogenesis in yeast: Sorting out the proteins. *Cell* **83**, 513-516.

Conradt, B., Haas, A. and Wickner, W. (1994). Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. *J. Cell Biol.* **126**, 99-110.

Cooper, A.A and Stevens, T.H. (1996). Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. *J. Cell Biol.* **133**, 529-541.

Cowles, C.R., Snyder, W.B., Burd, C.G. and Emr, S.D. (1997a). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. *EMBO J*. **16**, 2769-2782.

Cowles, C.R., Odorizzi, G., Payne, G.S. and Emr, S.D. (1997b). The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell* **91**, 109-118.

Craig, E.A. and Jacobsen, K. (1985). Mutations in cognate genes of *Saccharomyces cerevisiae* hsp70 result in reduced growth rates at low temperatures. *Mol. Cell Biol*. **5**, 3517-3524.

Cueva, R., Garcia-Alvarez, N. and Suarez-Rendueles, P. (1989). Yeast vacuolar aminopeptidase ysc I. Isolation and regulation of the *APE1* (*LAP4*) structual gene. *FEBS Lett*. **259**, 125-129.

Cyr, D.M., Lu, X. and Douglas, M.G. (1992). Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem*. **267**, 20927-20931.

Darsow, T., Rieder, S.E. and Emr, S.D. (1997). A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J. Cell Biol*. **138**, 517-529.

Dell'Angelica, E.C., Ohno, H., Eng Ooi, C., Rabinovich, E., Roche, K.W. and Bonifacino, J.S. (1997). AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J*. **16**, 917-928.

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. and Schekman, R. (1988). A subfamily of stress proteins facilitate translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**, 800-805.

Dice, J.F., Terlecky, S.R., Chiang, H.L., Olson, T.S., Isenman, L.D., Short-Russel, S.R., Freundlieb, S. and Terlecky, I.J. (1990). A selective pathway for degradation of cytosolic proteins by lysosomes. *Semin. Cell Biol*. **1**, 449-455.

Dulic, V. and Riezman, H. (1989). Characterisation of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. *EMBO J*. **8**, 1349-1359.

Dunn, W.A. (1994). Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends in Cell Biol*. **4**, 139-143.

Egner, R., Thumm, M., Straub, M., Simeon, A., Schüller, H.J. and Wolf, D.H. (1993). Tracing intracellular proteolytic pathways. Proteolysis of fatty acid synthase and other cytoplasmic proteins in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 27269-27276.

Ferro-Novick, S. and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* **370**, 191-193.

Flaherty, K.M., DeLuca-Flaherty, C. and McKay, D.B. (1990). Three dimensional structure of the ATPase fragment of a 70K heat shock cognate protein. *Nature* **346**, 623-628.

Flajnik, M., Canel, C., Kramer, J. and Kasahara, M. (1991). Hypothesis: Which came first, MHC class I or class II? *Immunogenetics* **33**, 295-300.

Flynn, G.C., Chappell, T.G. and Rothman, J.E. (1989). Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* **245**, 385-390.

Fourie, A.M., Sambrook, J.F. and Gething, M.J. (1994). Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J. Biol. Chem*. **269**, 30470-30478.

Freeman, B.C., Myers, M.P., Schumacher, R. and Morimoto, R.I. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J*. **14**, 2281-2292.

Frey, J. and Röhm, K.H. (1978). Subcellular localization and levels of aminopeptidases and dipeptidase in *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta*. **527**, 31-41.

Frydman, J. and Höhfeld, J. (1997). Chaperones get in touch: the Hip-Hop connection. *TIBS* **22**, 87-92.

Funakoshi, T., Matsuura, A., Noda, T. and Ohsumi, Y. (1997). Analyses of *APG13* gene involved in autophagy in yeast, *Saccharomyces cerevisiae*. *Gene* **192**, 207-213.

Gaut, J.R. and Hendershot, L.M. (1993). Mutations within the nucleotide binding site of immunoglobulin-binding protein inhibit ATPase activity and interfere with release of immunoglobulin heavy chain. *J. Biol. Chem.* **268**, 7248-7255.

George, M.D., Baba, M., Scott, S.V., Mizushima, N., Garrison, B.S., Ohsumi, Y. and Klionsky, D.J. (2000). Apg5p functions in the sequestration step in the cytoplasm-to-vacuole targeting and macroautophagy pathways. *Mol. Biol. Cell* **11**, 969-982.

Gething, M.J. and Sambrook, J. (1992). Protein folding in the cell. *Nature*, **355**, 33-45.

Glover, J.R., Andrews, D.W. and Rachubinski, R.A. (1994). *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA* **91**, 10541-10545.

Greene, L.E., Zinner, R., Naficy, S. and Eisenberg, E. (1995). Effect of nucleotide on the binding of peptides to 70-kDa heat shock protein. *J. Biol. Chem*. **270**, 2967-2973.

Gross, M. and Hessefort, S. (1996). Purification and characterisation of a 66-kDa protein from rabbit reticulocyte lysate which promotes the recycling of hsp70. *J. Biol. Chem*. **271**, 16833-16841.

Gross, T. and Schulz-Harder,B. (1986). *FEMS Microbiol. Lett*. **33**, 199-203.

Gutherie and Fink (1991). Methods in Enzymology 194, 15.

Haas, A. (1995). A quantitative assay to measure homotypic vacuole fusion invitro. *Methods in Cell Science* **17**, 283-294.

Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G. and Lithgow, T. (1995). Reconstitution of the initial steps of mitochondrial protein import. *Nature* **376**, 705-709.

Harding, T.M., Hefner-Gravink, A., Thumm, M. and Klionsky, D.J. (1996). Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. *J. Biol. Chem*. **271**, 17621-17624.

Hartl, F.U., Martin, J. and Neupert, W. (1992). Protein folding in the cell: the role of molecular chaperones Hsp70 and Hsp60. *Ann. Rev. Biophys. Biomol. Struct*. **21**, 293-322.

Hasilik, A. and Tanner, W. (1978). Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of the precursor into the enzyme. *Eur. J. Biochem*. **85**, 599-608.

Hendrick, J.P. and Hartl, F.U. (1995). The role of molecular chaperones in protein folding. *FASEB J*. **9**, 1559-1569.

Hendrick, J.P., Langer, T., Davis, T.A., Hartl, F.U. and Wiedmann, M. (1993). Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10216-10220.

Höhfeld, J. and Jentsch, S (1997). GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. *EMBO J*. **16**, 6209-6216.

Höhfeld, J., Minami, Y. and Hartl, F-U. (1995). Hip, a novel co-chaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell* **83**, 589-598.

Horazdovsky, B.F., DeWald, D.B. and Emr, S.D. (1995). Protein transport to the yeast vacuole. *Curr. Opi. In Cell Biol*. **7**, 544-551.

Horst, M., Oppliger, W., Rospert, S., Schönfeld, H.J., Schatz, G. and Azem, A. (1997). Sequential action of two hsp70 complexes during protein import into mitochondria. *EMBO J*. **16**, 1842-1849.

Huang, P.H. and Chiang, H.L (1997). Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. *J. Cell Biol*. **136**, 803-810.

Ikeda, E., Yoshida, S., Mitsuzawa, H., Uno, I. and Toh-e, A. (1994). YGE1 is a yeast homologue of Escherichia coli GrpE and is required for maintenance of mitochondrial functions. *FEBS Lett*. **339**, 265-268.

Jiang, R., Gao, B., Prasad, K., Greene, L.E. and Eisenberg, E. (2000). Hsc70 chaperones clathrin and primes it to interact with vesicle membranes. *J. Biol. Chem*. **275**, 8439-8447.

Kelly, R.B. (1985). Pathways of protein secretion in eukaryotes.*Science* **230**, 25-32.

Kim, J., Dalton, V.M., Eggerton, K.P., Scott, S.V. and Klionsky, D.J. (1999). Apg7p/ Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways. *Mol. Biol. Cell* **10**, 1337-1351.

Kim, J., Scott, S.V., Oda, M.N. and Klionsky, D.J. (1997). Transport of a large oligomeric protein by the cytoplasm to vacuole protein targeting pathway. *J. Cell Biol.* **137**, 609-618.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J. Cell Biol*. **147**, 435-446.

Klionsky, D.J. and Ohsumi, Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 1-32.

Klionsky, D.J., Cueva, R. and Yaver, D.S. (1992). Aminopeptidase I of *Saccharomyces cerevisiae* is localised to the vacuole independent of the secretory pathway. *J. Cell Biol.* **119**, 287-299.

Klionsky, D.J., Herman,P.K. and Emr, S.D. (1990). The fungal vacuole: composition, function and biogenesis. *Microbiol. Rev*. **54**, 266-292.

Klionsky, D.J. and Emr, S.D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J*. **8**, 2241-2250.

Klionsky, D.J., Banta, L.M. and Emr, S.D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. *Mol. Cell Biol*. **8**, 2105-2116.

Konopka, J.B., Jenness, D.D. and Hartwell, L.H. (1988). The C-terminus of the *S. cerevisiae* alpha-pheromone receptor mediates an adaptive response to pheromone. *Cell* **54**, 609-620.

Kronidou, N.G., Oppliger, W., Bolliger, L., Hannavy, K., Glick, B.S., Schatz, G. and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12818-12822.

Laloraya, S., Gambill, B.D. and Craig, E.A. (1994). A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6481-6485.

Lang, T., Schaeffeler, E., Bernreuther, D., Bredschneider, M., Wolf, D.H. and Thumm, M. (1998). Aut2p and Aut7p, two novel microtubule-associated proteins are essential for delivery of autophagic vesicles to the vacuole. *EMBO J.* **17**, 3597-3607.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. and Hartl, F.U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356**, 683-689.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2874-2878.

Lindquist, S. and Craig, E.A. (1988). The heat-shock proteins. *Ann. Rev. Genet.* **22**, 631-677.

Lindquist, S. (1986). The heat shock response. *Ann. Rev. Biochem*. **55**, 1151-1191.

Marshall, J.S., DeRocher, A.E., Keegstra, K. and Vierling, E. (1990). Identification of heat shock protein hsp70 homologs in chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 374-378.

Martinez, E., Segui-Real, B., Silles, E., Mazon, M.J. and Sandoval, I.V. (1999). The prepropeptide of vacuolar aminopeptidase I is necessary and sufficient to target the fluorescent reporter protein GFP to the vacuole of yeast by the Cvt pathway. *Mol. Microbiol*. **33**, 52-62.

Martinez, E., Jimenez, M.A., Segui-Real, B., Vandekerckhove, J. and Sandoval, I.V. (1997). Folding of the presequence of yeast pAPI into an amphipathic helix determines transport of the protein from the cytosol to the vacuole. *J. Mol. Biol*. **267**, 1124-1138.

Matile, P., Wiemken, A. and Guyer, W. (1971). A lysosomal aminopeptidase isozyme in differentiating yeast cells and protoplasts. *Planta*. **96**, 43-53.

Mayer, A. and Wickner, W. (1997). Docking of yeast vacuoles is catalysed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J. Cell Biol.* **136**, 307-317.

Mayer, A., Wickner, W. and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (α-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**, 83-94.

Mazon, M.J., Gancedo, J.M. and Gancedo, C. (1982). Inactivation of yeast fructose-1,6-bisphosphatase. In vivo phosphorylation of the enzyme. *J. Biol. Chem*. **257**, 1128-1130.

McCarty, J.S., Buchberger, A., Reinstein, J. and Bukau, B. (1995). The role of ATP in the functional cycle of the DnaK chaperone system. *J. Mol. Biol.* **249**, 126-137.

McKay, D.B. (1991). Structure of the 70-kilodalton heat-shock-related proteins. *Springer Semin. Immunopathol*. **13**, 1-9.

McNew, J.A. and Goodman, J.M. (1994). An oligomeric protein is imported into peroxisomes in vivo. *J. Cell Biol*. **127**, 1245-1257.

Mechler, B., Hirsch, H.H., Müller, H. and Wolf, D.H. (1988). Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. *EMBO J*. **7**, 1705-1710.

Mechler, B., Müller, H. and Wolf, D.H. (1987). Maturation of vacuolar (lysosomal) enzymes in yeast: proteinase yscA and proteinase yscB are catalysts of the processing and activation event of carboxypeptidase yscY. *EMBO J*. **6**, 2157-2163.

Mechler, B., Müller, M., Müller, H., Muessdoerffer, F. and Wolf, D.H. (1982). In vivo biosynthesis of the vacuolar proteinases A and B in the yeast *Saccharomyces cerevisiae. J. Biol. Chem.* **257**, 11203-11206.

Metz, G. and Röhm, K.H. (1976). Yeast aminopeptidase I. Chemical composition and catalytic properties. *Biochem. Biophys. Acta*. **429**, 933-949.

Minami, Y., Höhfeld, J., Ohtsuka, K. and Hartl, F.U. (1996). Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. *J. Biol. Chem*. **271**, 19617-19624.

Mizushima, N., Noda, T. and Ohsumi, Y. (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *EMBO J*. **18**, 3888-3896.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M. and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature* **395**, 395-398.

Moehle, C.M., Dixon, C.K. and Jones, E.W. (1989). Processing pathway for protease B of *Saccharomyces cerevisiae. J. Cell Biol.* **108**, 309-325.

Mortimore, G.E., Poso, A.R. and Lardeux, B.R. (1989). Mechanism and regulation of protein degradation in liver. *Diabetes Metab. Rev.* **5**, 49-70.

Mortimore, G.E., Lardeux, B.R. and Adams, C.E. (1988). Regulation of microautophagy and basal protein turnover in rat liver. Effects of short term starvation. *J. Biol. Chem.* **263**, 2506-2512.

Nakai, M., Kato, Y., Ikeda, E., Toh-e, A. and Endo, T. (1994). Yge1p, a eukaryotic GrpE homolog, is localised in the mitochondrial matrix and interacts with mitochondrial hsp70. *Biochem. Biophys. Res. Commun*. **200**, 435-442.

Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. and Craig, E.A. (1992). The translation machinery and 70 kD heat shock protein cooperate in protein synthesis. *Cell* **71**, 97-105.

Nelson, H. and Nelson, N. (1990). Disruption of genes encoding subunits of yeast vacuolar H^+- ATPase causes conditional lethality. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3503-3507.

Newman, L.S., Mckeever, M.O., Okano, H.J. and Darnell. R.B. (1995). beta-NAP, a cerebellar degeneration antigen, is a neuron specific vesicle coat protein. *Cell* **82**, 773-783.

Nguyen, T.H., Law, D.T. and Williams, D.B. (1991). Binding protein BiP is required for translocation of secretory proteins into the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci*. **88**, 1565-1569.

Nichols, B.J., Ungermann, C., Pelham, H.R.B., Wickner, W. and Haas, A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* **387**, 199-202.

Noda, T., Kim, J., Huang, W-P., Baba, M., Tokunaga, C., Ohsumi, Y. and Klionsky, D.J. (2000). Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J. Cell Biol*. **148**, 465-479.

Noda. T. and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem*. **273**, 3963-3966.

Noda T., Matsuura, A., Wada, Y. and Ohsumi, Y. (1995). Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun*. **210**, 126-132.

Oda, M.N., Scott, S.V., Hefner-Gravink, A., Caffarelli, A.D. and Klionsky, D.J. (1996). Identification of a cytoplasm to vacuole targeting determinant in aminopeptidase I. J. Cell Biol. 132, 999-1010.

Palleros, D.R., Reid, K.L., Shi, L., Welch, W.J. and Fink, A.L. (1993). ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. *Nature* **365**, 664-666.

Parsell, D.A., Taulien, J. and Lindquist, S. (1993). The role of heat-shock protein in thermotolerance. *Philos. Trans. R. Soc: Lond. B. Biol. Sci*. **339**, 279-286.

Pelham, H.R. (1986). Speculations on the functions of the major heat shock and glucose regulated protiens. *Cell* **46**, 959-961.

Peters, C., Andrews, P.D., Stark, M.J.R., Cesaro-Tadic, S., Glatz, A., Podtelejnikov, A., Mann, M. and Mayer, A. (1999). Control of the terminal step of intracellular membrane fusion by protein phosphatase 1. *Science* **285**, 1084-1087.

Peters, C. and Mayer, A. (1998). Ca2+/ calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature* **396**, 575-580.

Pfeffer, S.R, (1988). Mannose 6-phosphate receptors and their role in targeting proteins to lysosomes. *J. Membr. Biol*. **103**, 7-16.

Piper, R.C., Bryant, N.J. and Stevens, T.H. (1997). The membrane protein Alkaline Phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. *J. Cell Biol*. **138**, 531-545.

Piper, R.C., Cooper, A.A., Yang, H. and Stevens, T.H. (1995). *VPS27* controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae. J. Cell Biol.* **131**, 603-617.

Plemper, R.K., Böhmler, S., Bordallo, J., Sommer, T. and Wolf, D.H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388**, 891-895.

Pohlig, G. and Holzer, H. (1985). Phosphorylation and inactivation of yeast fructose-1,6-bisphosphatase by cyclic AMP-dependent protein kinase from yeast. *J. Biol. Chem.* **260**, 13818-13823.

Preston, R.A., Manolson, M.F., Becherer, K., Weidenhammer, E., Kirkpatrick, D., Wright, R. and Jones, E.W. (1991). Isolation and characterisation of *PEP3*, a gene required for vacuolar biogenesis in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **11**, 5801-5812.

Price, A., Seals, D., Wickner, W. and Ungermann, C. (2000). The docking stage of yeast vacuole fusion requires the transfer of proteins from a cis-SNARE complex to a Rab/Ypt protein. *J. Cell Biol*. **148**, 1231-1238.

Prip-Buus, C., Westerman, B., Schmitt, M., Langer, T., Neupert, W. and Schwarz, E. (1996). Role of the mitochondrial DnaJ homologue, Mdj1p, in the prevention of heat-induced protein aggregation. *FEBS Lett*. **380**, 142-146.

Purwin, C., Nicolay, K., Scheffers, W.A. and Holzer, H. (1986). Mechanism of control of adenylate cyclase activity in yeast by fermentable sugars and carbonyl cyanide m-chlorophenylhydrazone. *J. Biol. Chem*. **261**, 8744-8749.

Rachubinski, R.A. and Subramani, S. (1995). How proteins penetrate peroxisomes. *Cell* **83**, 525-528.

Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996). Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Ann. Rev. Biochem*. **65**, 271-303.

Rassow, J., Maarse, A.C., Krainer, E., Kübrich, M., Müller, H., Meijer, M., Craig, E.A. and Pfanner, N. (1994). Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J. Cell Biol*. **127**, 1547-1556.

Raymond, C.K., Roberts, C.J., Moore, K.E., Howald, I. and Stevens, T.H. (1992a). Biogenesis of the vacuole in *Saccharomyces cerevisiae*. *Int. Rev. of Cytology* **139**, 59-120.

Raymond, C.K., Howald-Stevenson, I., Vater, C. and Stevens, T.H. (1992b). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell* **3**, 1389-1402.

Richarme, G. and Kohiyama, M. (1993). Specificity of the *Escherichia coli* chaperone DnaK (70-kDa heat shock protein) for hydrophobic amino acids. *J. Biol. Chem*. **268**, 24074-24077.

Rippmann, F., Taylor, W.R., Rothbard, J.B. and Green, N.M. (1991). A hypothetical model for the peptide binding domain of hsp70 based on the peptide binding domain of HLA. *EMBO J.* **10**, 1053-1059.

Robinson, J.S., Graham, T.R. and Emr, S.D. (1991). A putative zinc finger protein, *Saccharomyces cerevisiae* Vps18p, affects late Golgi functions required for vacuolar protein sorting and efficient α-factor prohormone maturation. *Mol. Cell Biol*. **11**, 5813-5824.

Rothblatt, J., Deshaies, R., Sanders, S., Daum, G. and Schekman, R. (1989). Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum. *J. Cell Biol*. **109**, 2641-2652.

Rothman, J.E. and Wieland, F.T. (1996). Protein sorting by transport vesicles. *Science* **272**, 227-234.

Rothman, J.E. (1994). Mechanisms of intracellular membrane fusion. *Nature* **372**, 55-63.

Rothman, J.H. and Stevens, T.H. (1986). Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell* **47**, 1041-1051.

Rowley, N., Prip-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barrell, B. and Neupert, W. (1994). Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. *Cell* **77**, 249-259.

Satyanarayana, C., Schröder-Köhne, S., Craig, E.A., Schu, P.V. and Horst, M. (2000). Cytosolic Hsp70s are involved in the transport of aminopeptidase 1 from the cytoplasm into the vacuole. *FEBS Lett.* **470**, 232-238.

Satyanarayana, C. and Horst. M. (1998). The ATP-driven protein translocation-motor of mitochondria. Gene Therapy and Molecular Biology. Vol.1, 741-748. Published by Gene Therapy Press, Palo Alto, CA.

Schatz, G. and Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science* **271**, 1519-1526.

Schekman, R. (1998). Membrane fusion. Ready...aim...fire! *Nature* **396**, 514-515.

Schmid, D., Baici, A., Gehring, H. and Christen, P. (1994). Kinetics of molecular chaperone action. *Science* **263**, 971-973.

Schneider, H.C., Berthold, J., Bauer, M.F., Dietmeier, K., Guiard, B., Brunner, M. and Neupert, W (1994). Mitochondrial HSP70/ MIM44 complex facilitates protein import. *Nature* **371**, 768-774.

Schnell, D.J., Kessler, F. and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. *Science* **266**, 1007-1012.

Schork, S.M., Thumm, M. and Wolf, D.H. (1995). Catbolite inactivation of fructose-1,6-bisphosphatase of *Saccharomyces cerevisiae*. Degradation occurs via the ubiquitin pathway. *J. Biol. Chem*. 270, 26446-26450.

Schröder, H., Langer, T., Hartl, F.U. and Bukau, B. (1993). Dnak, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* **12**, 4137-4144.

Schüle, T., Rose, M., Entian, K.D., Thumm, M. and Wolf, D.H. (2000). Ubc8p functions in catabolite degradation of fructose-1,6-bisphosphatase in yeast. *EMBO J.* **19**, 2161-2167.

Schworer, C.M., Shiffer, K.A. and Mortimore, G.E. (1981). Quantitative relationship between autophagy and proteolysis during graded amino acid deprivation in perfused rat liver. *J. Biol. Chem.* **256**, 7652-7658.

Scott, S.V., Nice, D.C 3rd., Nau, J.J., Weisman, L.S., Kamada, Y., Keizer-Gunn, I., Funakoshi, T., Veenhuis, M., Ohsumi, Y. and Klionsky, D.J. (2000). Apg13p and Vac8p are part of a complex of phosphoprotein that are required for cytoplasm to vacuole targeting. *J. Biol. Chem*. **275**, 25840-25849.

Scott, S.V., Baba, M., Ohsumi, Y. and Klionsky, D.J. (1997). Aminopeptidase I is targeted to the vacuole by a nonclassical vesicular mechanism. *J. Cell Biol*. **138**, 37-44.

Scott, S.V., Hefner-Gravink, A., Morano, K.A., Noda, T., Ohsumi, Y. and Klionsky, D.J. (1996). Cytoplasm to vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. *Proc. Natl. Acad. Sci. USA* **93**, 12304-12308.

Seguí-Real, B., Martinez, M. and Sandoval, I.V. (1995). Yeast aminopeptidase I is post translationally sorted from the cytosol to the vacuole by a mechanism mediated by its bipartite N-terminal extension. *EMBO J.* **14**, 5476-5484.

Silles, E., Mazon, M.J., Gevaert, K., Goethals, M., Vandekerckhove, J., Leber, R. and Sandoval, I.V. (2000)
Targeting of aminopeptidase I to the yeast vacuole is mediated by Ssa1p, a cytosolic member of the 70 kD stress protein familly.
J. Biol. Chem. July 19; [epub ahead of print]

Simpson, F., Peden, A.A., Christopoulou, L. and Robinson, M.S. (1997). Characterisation of the adaptor-related protein complex, AP-3. *J. Cell Biol.* **137**, 835-845.

Simpson, F., Bright, N.A., West, M.A., Newman, L.S., Darnell, R.B. and Robinson, M.S. (1996). A novel adaptor-related protein complex. *J. Cell Biol*. **133**, 749-760.

Singer, B. and Riezman, H. (1990). Detection of an intermediate compartment involved in transport of alpha factor from the plasma membrane to the vacuole in yeast. *J. Cell Biol*. **110**, 1911-1922.

Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990). The *E. coli DnaK* gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* **62**, 939-944.

Stevens, T.H., Rothman, J.H., Payne, G.S. and Schekman, R. (1986). Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol*. **102**, 1551-1557.

Subramani, S. (1993). Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol*. **9**, 445-478.

Szabo, A., Langer, T., Schröder, H., Flanagan, J., Bukau, B. and Hartl, F.U. (1994). The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system DnaK, DnaJ and GrpE. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10345-10349.

Takeshige, K., Baba, M., Tsuboi, S., Noda, T. and Ohsumi, Y. (1992). Autophagy in yeast demonstrated with proteinase–deficient mutants and conditions for its induction. *J. Cell Biol*. **119**, 301-311.

Teichert, U., Mechler, B., Müller, H. and Wolf, D.H. (1989). Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J. Biol. Chem*. **264**, 16037-16045.

Terlecky, S.R., Chiang, H.L., Olson, T.S. and Dice, J.F. (1992). Protein and peptide binding and stimulation of *in vitro* lysosomal proteolysis by the 73 kDa heat shock cognate protein. *J. Biol. Chem.* **267**, 9202-9209.

Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M. and Wolf, D.H. (1994). Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett*. **349**, 275-280.

Toledo, H., Carlino, A., Vidal, V., Redfield, B., Nettleton, M.Y., Kochan, J.P., Brot, N. and Weissbach, H. (1993). Dissociation of glucose-regulated protein Grp78 and Grp78-IgE Fc complexes by ATP. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2505-2508.

Trumbly, R.J. and Bradley, G. (1983). Isolation and characterisation of aminopeptidase mutants of *Saccharomyces cerevisiae*. *J. Bacteriol*. **156**, 36-48.

Tsukada, M. and Ohsumi, Y. (1993). Isolation and characterisation of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett*. **333**, 169-174.

Ungermann, C., Fischer von Mollard, G., Jensen, O.N., Margolis, N., Stevens, T.H. and Wickner, W. (1999a). Three v-SNAREs and two t-SNAREs, present in a pentameric cis-SNARE complex on isolated vacuoles, are essential for homotypic vacuole fusion. *J. Cell Biol*. **145**, 1435-1442.

Ungermann, C., Wickner, W. and Xu, Z. (1999b). Vacuole acidification is required for trans-SNARE pairing, LMA1 release, and homotypic fusion. *Proc. Natl. Acad. Sci. USA* **96**, 11194-11199.

Ungermann,C. and Wickner, W. (1998). Vam7p, a vacuolar SNAP-25 homolog, is required for SNARE complex integrity and vacuole docking and fusion. *EMBO J*. **17**, 3269-3276.

Ungermann, C., Sato, K. and Wickner, W. (1998). Defining the functions of trans-SNARE pairs. *Nature* **396**, 543-548.

Van Den Hazel, H.B., Kielland-Brandt, M.C. and Winther, J.R. (1992). Autoactivation of proteinase A initiates activation of yeast vacuolar zymogens. *Eur. J. Biochem*. **207**, 227-283.

Vogel, J.P., Misra, L.M. and Rose, M.D. (1990). Loss of BiP/ GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol*. **110**, 1885-1895.

Wagner, I., Arlt, H., van Dyck, L., Langer, T. and Neupert, W. (1994). Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *EMBO J*. **13**, 5135-5145.

Wawrzynow, A., Banecki, B., Wall, D., Liberek, K., Georgopoulos, C. and Zylicz, M. (1995). ATP hydrolysis is required for the DnaJ-dependent activation of DnaK chaperone for binding to both native and denatured protein substrates. *J. Biol. Chem*. **270**, 19307-19311.

Weis, W.I. and Scheller, R.H. (1998). Membrane fusion. SNARE the rod, coil the complex. *Nature* **395**, 328-329.

Werner-Washburne, M., Stone, D.E. and Craig, E.A. (1987). Complex interactions among members of an essential subfamily of HSP70 genes in *Saccharomyces cerevisiae*. *Mol. Cell Biol*. **7**, 2568-2577.

Westermann, B., Gaume, B., Herrmann, J.M., Neupert, W. and Schwarz, E. (1996). Role of the mitochondrial DnaJ homolog Mdj1p as a chaperone for mitochondrially synthesized and imported proteins. *Mol. Cell Biol*. **16**, 7063-7071.

Westphal, V., Marcusson, E.G., Winther, J.R., Emr, S.D, and van den Hazel, H.B. (1996). Multiple pathways for vacuolar sorting of yeast proteinase A. *J. Biol. Chem*. **271**, 11865-11870.

Winther, J.R. (1989). PhD. Thesis, Carlsberg Lab., Copenhagen.

Wolf, A.M., Din, N. and Petersen, J.G. (1996). Vacuolar and extracellular maturation of *Saccharomyces cerevisiae* proteinase A. *Yeast* **12**, 823-832.

Wolf, D.H. and Fink, G.R. (1975). Proteinase C (carboxypeptidase Y) mutant of yeast. *J. Bacteriol*. **123**, 1150-1156

Xu, Z., Sato, K. and Wickner, W. (1998). LMA1 binds to vacuoles at Sec18p (NSF), transfers upon ATP hydrolysis to a t-SNARE (Vam3p) complex, and is released during fusion. *Cell* **93**, 1125-1134.

Yamashiro, C.T., Kane, P.M., Wolczyk, D.F., Preston, R.A. and Stevens, T.H. (1990). Role of vacuolar acidification in protein sorting and zymogen activation: a genetic analysis of the yeast vacuolar proton-translocating ATPase. *Mol. Cell Biol*. **10**, 3737-3749.

Yoshihisa, T. and Anraku, Y. (1990). A novel pathway of import of α-mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae. J. Biol. Chem*. **265**, 22418-22425

Zhong, T. and Arndt, K.T. (1993). The yeast SIS1 protein, a DnaJ homologue, is required for the initiation of translation. *Cell* **73**, 1175-1186.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E. and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606-1614.

Ziegelhoffer, T., Lopez-Buesa, P. and Craig, E.A. (1995). The dissociation of ATP from hsp70 of *Saccharomyces cerevisiae* is stimulated by both Ydj1p and peptide substrates. *J. Biol. Chem*. **270**, 10412-10419.

Zubenko, G.S. and Jones, E.W. (1981). Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. *Genetics* **97**, 45-64.

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Lebenslauf

Am 12.12.1972 wurde ich als drittes Kind von Frau Krishnaveni Satyanarayana und Herrn N. Satyanarayana in Mysore, Indien geboren. Ich besitze die indische Staatsangehörigkeit.

Von 1977-1982 besuchte ich die Grundschule in Bhubaneshwar (Indien). Anschließend war ich Schülerin der C.F.T.R.I. Schule in Mysore wo ich 1989 meinen Schulabschluss machte.

Mein voruniversitäres Studium absolvierte ich von 1989 bis 1991 am Sarada Vilas College in Mysore.

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Ebenfalls an der Universität von Mysore erhielt ich 1996 den Titel Master of Science in Biotechnologie. Meine Studienarbeit im Rahmen des Masterprogramms fertigte ich am National Centre for Biological Sciences, Tata Institute of Fundamental Research in Bangalore, Indien an. Dort arbeitete ich zusammen mit Dr. Satyajit Mayor und entwickelte eine Primärzellkultur von frühen Embryos von *Drosophila melanogaster* zur Analyse der Endozytose.

Nach meinem Master-Abschluss 1996 arbeitete ich acht Monate als Nachwuchswissenschaftler bei Dr. Satyajit Mayor. Während dieser Zeit stellte ich ein Fusionskonstrukt zwischen dem Folat- und dem low-density-lipoprotein-Rezeptor her.

Im April 1997 begann ich meine Doktorarbeit am Institut für Biochemie II in Göttingen unter der Leitung von Prof. Dr. K. von Figura. Der Titel meiner Arbeit lautet: „Requirement of HSP70s in the cytosol to vacuole transport of Aminopeptidase 1 in *Saccharomyces cerevisiae* “.