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Cytosolic Hsp70s are involved in the transport of aminopeptidase 1 from the cytoplasm into the vacuole

Chitkala Satyanarayana[a], Stephan Schröder-Köhne[b], Elizabeth A. Craig[c], Peter V. Schu[a,*], Martin Horst[a]

[a]*Institut für Molekulare Zellbiologie und Biochemie, Abteilung Biochemie II, Universität Göttingen, Heinrich-Düker Weg 12, D-37073 Göttingen, Germany*
[b]*Max-Planck Institute of Biophysical Chemistry, Am Faßberg 11, D-37077 Göttingen, Germany*
[c]*Department of Biomolecular Chemistry, University of Wisconsin Medical School, 587 Medical Sciences Building, 1300 University Avenue, Madison, WI 53706-1532, USA*

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Abstract Eukaryotic 70 kDa heat shock proteins (Hsp70s) are localized in various cellular compartments and exhibit functions such as protein translocation across membranes, protein folding and assembly. Here we demonstrate that the constitutively expressed members of the yeast cytoplasmic Ssa subfamily, Ssa1/2p, are involved in the transport of the vacuolar hydrolase aminopeptidase 1 from the cytoplasm into the vacuole. The Ssap family members displayed overlapping functions in the transport of aminopeptidase 1. In *SSA1* and *SSA11* deletion mutants the precursor of aminopeptidase 1 accumulated in a dodecameric complex that is packaged in prevacuolar transport vesicles. Ssa1/2p was prominently localized to the vacuolar membrane, consistent with the role we propose for Ssa proteins in the fusion of transport vesicles with the vacuolar membrane.

Key words: Aminopeptidase 1; Heat shock protein 70; Protein transport; Vacuole; Yeast

1. Introduction

Cytosolic members of the 70 kDa heat shock protein family (Hsp70) facilitate import of precursor proteins into chloroplasts, into the endoplasmic reticulum (ER) and into mitochondria [1–4]. They are also involved in the transport of proteins into peroxisomes and nuclei [5,6]. Cytosolic Hsp70s also appear to be involved in the transport of a certain subclass of cytoplasmic proteins into lysosomes of mammalian cells [7]. However, the precise function of Hsp70s in these import pathways is not known.

Other members of the Hsp70 family are located in the lumen of chloroplasts, ER, lysosomes and mitochondria. Besides their role in protein folding they are also involved in the transport of precursor proteins across the membranes of ER, chloroplasts and mitochondria [4,8–14].

Six members of the Hsp70 family are present in the cytoplasm of *Saccharomyces cerevisiae* (Ssa1p, Ssa2p, Ssa3p, Ssa4p, Ssb1p, Ssb2p [15]). Ssa proteins (Ssap) are known to be involved in protein import into organelles and in the assembly of oligomeric protein complexes, whereas the Ssb proteins are involved in protein biosynthesis. Ssa1p and Ssa2p are constitutively expressed, Ssa3p and Ssa4p are expressed under stress conditions.

In *S. cerevisiae* soluble resident vacuolar proteins are transported to the vacuole by at least two different pathways: through the classical secretory pathway as well as through the direct cytoplasm to vacuole transport pathway. Most of the vacuolar resident proteins, such as carboxypeptidase Y (CPY) and proteinase A, first enter the ER and travel through the Golgi from where they are transported into vesicles and to the vacuole [16–18]. α-Mannosidase and aminopeptidase 1 (Ape1p) are examples of resident vacuolar proteins which are transported to the vacuole directly from the cytoplasm [19,20].

Aminopeptidase 1 is synthesized as a 61 kDa cytoplasmic precursor protein pApe1p [21]. The maturation of pApe1p is a two-step process occurring in the vacuole. The 61 kDa precursor is sequentially converted into a 55 kDa intermediate by proteinase A and finally into the 50 kDa mature form by proteinase B [22]. The active enzyme is a dodecameric complex with a molecular mass of approximately 600 kDa [23]. Transport of the precursor protein to the vacuole occurs independently of the secretory pathway. Several independent pieces of evidence support this conclusion: pApe1p transport is not affected in mutants of the early secretory and Golgi to vacuole pathway. Overexpression of vacuolar hydrolases leads to their secretion, whereas overexpressed Ape1p is not secreted [20]. pApe1p does not contain an ER signal sequence [24]. The targeting signal resembles much more a mitochondrial targeting signal [25,26]. pApe1p has a half-life of maturation of 45 min compared to a half-life of approximately 6 min for other vacuolar hydrolases transported via the secretory pathway [20].

Two different mechanisms might be operative in pApe1p transport [22,27,28]. A translocation mechanism and a vesicle-mediated process have been proposed. Seguí-Real and coworkers [22] showed that a modified pApe1p carrying a myc tag at the C-terminus got stuck in the vacuolar membrane. The vacuolar targeting signal of this stuck import intermediate was processed to the intermediate form by proteinase A. The myc epitope was sensitive to externally added proteinase in the absence of a detergent, suggesting that the

*Corresponding author. Fax: (49)-551-395979.
E-mail: schu@ukb2-00.uni-bc.gwdg.de

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N-terminus of the stuck pApe1p is in the vacuolar lumen while the C-terminus is in the cytoplasm. Analysis of cytoplasm to vacuole targeting mutants [27–29] demonstrates a vesicle-based transport mechanism. The observation that the precursor of pApe1p assembles with a half-life of 2 min in the cytoplasm indicates that pApe1p enters the cytoplasm to vacuole (Cvt) vesicles as a pre-dodecamer. Cvt vesicles have a double membrane supporting the idea of an autophagocytosis-like mechanism. The genetic and phenotypic overlap between autophagy and the Cvt targeting pathway suggests that the pApe1p transport pathway uses some components of the autophagocytosis machinery.

It has been shown that proteins entering mitochondria or ER are partially unfolded [1,30–32]. In contrast, conformational studies of peroxisomal proteins have shown that they can enter the peroxisomal matrix in a fully folded state [33]. In fact, large pre-assembled oligomeric complexes can be imported into the peroxisomal lumen. Studies indicate that pApe1p enters the vacuole as a fully assembled pre-dodecamer [34]. Pulse chase experiments suggested that oligomerization is an early step in pApe1p import. Mutants in the Cvt pathway and propeptide deletion mutants are defective only in import and not in oligomerization suggesting that the propeptide is responsible for vacuolar delivery [25,26].

The N-terminal cleavable propeptide of pApe1p is 45 amino acids long and forms two α-helices separated by a β-turn. The presequence of 17 residues folds into an amphipathic α-helix similar to the mitochondrial targeting signals [20,25,26]. Using NMR spectroscopy it has been shown that mutations which destabilize the helix inhibit the transport of pApe1p [26].

The similarity of mitochondrial and vacuolar targeting sequences suggested that similar targeting factors are involved in the transport of proteins in both transport pathways. Therefore, we investigated the function of the cytoplasmic Hsp70 chaperones of the Ssap family in the transport of pApe1p into the vacuole.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strains used in this study were: MW109 (referred to as wild-type in this study): *MATa his3 leu2 lys2 Δtrp1 ura3*; JN114 (referred to as Δ*ssaI* in this study): *MATa his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa1::HIS3*; JN115 (referred to as Δ*ssaII* in this study): *MATa his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa2::URA3*; MW123 (referred to as Δ*ssaI/II* in this study): *MATa his3 leu2 lys2 Δtrp1 ura3 ssa1::HIS3 ssa2::LEU2*. The above described strains are described by Werner-Washburne et al. [15]. MH50 (referred to as Δ*ssaII+pSSAI* in this study): *MATa his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa2::URA3 Ycplac111-SSAI*; MH51 (referred to as Δ*ssaII+pSSAII* in this study): *MATa his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa2::URA3 Ycplac111-SSAII*; MH52 (referred to as Δ*ssaI+pSSAII* in this study): *MATa his3-11,3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 ssa1::HIS3 Ycplac111-SSAII*.

Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), SD-N (0.17% yeast nitrogen base without amino acids, 2% glucose; [43]), or SD (0.67% yeast nitrogen base without amino acids, 2% glucose). The powdered media were purchased from Gibco-BRL.

2.2. Protein extraction

Cells were grown to the mid-exponential phase in YPD and pelleted at 4500×*g* for 5 min. The cell pellet was resuspended in Tris–SO_4/DTT buffer (0.1 M Tris–SO_4 pH 9.4, 10 mM dithiothreitol) at a final concentration of 10 OD_{600} cells/ml. The cells were incubated at room temperature for 20 min, again pelleted at 1500×*g* for 5 min and resuspended in spheroplasting buffer (1.2 M sorbitol, 50 mM Tris–HCl pH 7.4, lyticase 2.5 μl/10 OD_{600} (Boehringer, Mannheim)) and incubated at 30°C for 30 min with slow shaking. Spheroplasts were pelleted at 4500×*g* for 3 min and resuspended in 700 μl of 0.1 M sodium phosphate pH 7.4. Spheroplasts were broken using glass beads. Samples were vortexed at maximum speed three times, 2 min each. Unbroken spheroplasts and glass beads were pelleted in a microfuge. The supernatant was collected and the protein content estimated using the Bio-Rad reagent (Bio-Rad, Hercules, CA). 70 μg of total protein was precipitated, resuspended in Laemmli buffer and separated by 10% SDS–PAGE.

2.3. Glycerol density gradients

Cell extracts were prepared as described above. 20–50% glycerol step gradients (20%, 30%, 40% and 50% glycerol solutions in 20 mM K-PIPES pH 6.8) were prepared as described by Scott et al. [37]. 1.2 mg of protein was loaded onto each gradient and centrifuged at 55 000 rpm for 4 h at 15°C using the TLS-55 rotor in the Beckmann table-top ultracentrifuge. Following centrifugation, 10 fractions were collected from each gradient, the proteins were precipitated in trichloroacetic acid (TCA), resuspended in Laemmli buffer and separated by 10% SDS–PAGE.

2.4. Isolation of vacuoles

Cell fractionation was essentially done as described by Scott et al. [37] with the following modifications. Cells were grown in YPD at 30°C to an OD_{600} of 0.8–1.0. Cells were harvested by a 5 min spin at 4500×*g*, incubated for 20 min at 30°C with 100 mM Tris–SO_4 pH 9.4, 10 mM DTT and pelleted for 5 min at 4500×*g*. The cell pellet was resuspended in spheroplasting buffer (1.2 M sorbitol, 50 mM Tris–HCl pH 7.4, 0.5 mg zymolyase 20T/50 OD_{600} (Seikagaku, Tokyo) and incubated for 30 min at 30°C. Spheroplasts were pelleted for 2 min at 4°C at 500×*g*. Spheroplasts were lysed with water and mixed with 60% Optiprep to give a final concentration of 37% in 3 ml 10 mM K-PIPES pH 6.8. The suspension was overlaid with 2 ml of 30, 25, 19, and 0% fractions and was centrifuged at 25 000 rpm for 4 h at 4°C in a SW40 (Beckman). For Ficoll gradients the equivalent of 300 OD_{600} of cells was used per gradient. The spheroplasting was done with zymolyase 20T (Seikagaku Corporation, Japan) at a concentration of 0.5 mg/50 OD_{600} of cells. The spheroplasts were lysed with water and mixed with 40% Ficoll, sorbitol (2.5 M stock) and K-PIPES pH 6.8 (100 mM stock) were added to final concentrations of 12%, 200 mM and 10 mM respectively. The resuspension was overlaid with 2 ml of 8% Ficoll, 2 ml 4% Ficoll and 1 ml of 200 mM sorbitol, 10 mM K-PIPES pH 6.8. The gradient was centrifuged at 30 000 rpm for 90 min at 4°C in the Beckmann SW40 rotor. Interphases were collected and analyzed by Western blotting. The protein content was estimated using the Bio-Rad protein assay reagent. A 500 ml culture yields approximately 350 μg vacuolar protein.

2.5. Protease treatment

Cells were spheroplasted and differentially lysed as described above. Proteinase K or proteinase K together with Triton X-100 was added to a final concentration of 50 μg/ml or 0.2%. The samples were incubated on ice for 30 min, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM and the reaction was further incubated for 10 min on ice. The proteins were TCA-precipitated, resuspended in Laemmli buffer and separated by 10% SDS–PAGE.

2.6. Immunofluorescence

Cells were grown to exponential phase in YPUAD at 25°C, spun down for 2 min at 2500×*g* and resuspended in fixative (3.5% PFA/10% sorbitol/PBS). Cells were fixed for 2 h at room temperature. Immunofluorescence was then performed as described [35]. After permeabilization of the cell wall with lyticase cells were quenched with PBS/10% sorbitol/1% BSA and then incubated with antiserum recognizing both Ssa1p and Ssa2p diluted 1:4000 in quenching solution for 1 h. Secondary Cy3-conjugated goat anti-rabbit antibodies were applied for 30 min.

2.7. Molecular biology

Ssa2p was reintroduced into JN115 and JN114 by transforming the strains with a derivative of the plasmid Ycplac111 containing a 5.5 kb genomic clone of *SSAII*. Ssa1p was reintroduced into JN114 and JN115 by transforming the strains with a derivative of the plasmid Ycplac111 containing a 6.5 kb genomic clone of *SSAI*.

3. Results

3.1. Ssa proteins are involved in the transport of aminopeptidase 1 from the cytoplasm into the vacuole

To test whether Ssa proteins are involved in the transport of pApe1p from the cytoplasm into the vacuole we used yeast strains in which the constitutively expressed Ssa1p, Ssa2p, or both are not present [15]. The steady-state level of the precursor and the mature form of Ape1p in these strains was determined by Western blotting. As the maturation of pApe1p occurs only inside the vacuole, the presence of the mature protein can be used as an indicator for the vacuolar delivery of the precursor [20].

Wild-type (wt) yeast cells and *SSA* deletion mutants were grown to mid-exponential phase in rich medium to prevent upregulation of autophagocytosis. Western blot analysis was done using a polyclonal anti-Ape1p antibody. In the wt strain only the mature form of Ape1p (mAPe1p) with a molecular weight of 50 kDa was detected (Fig. 1A, lane 1). In the strains

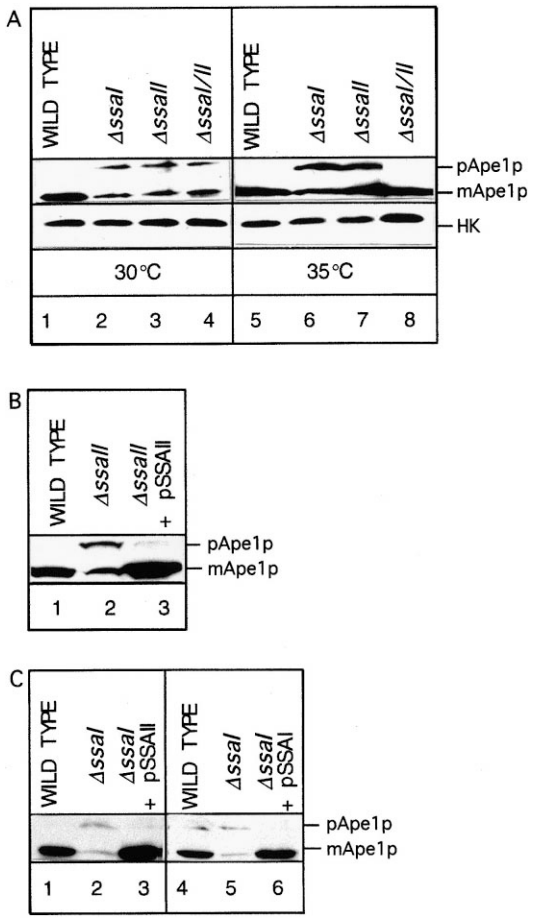

Fig. 1. Accumulation of Ape1p precursor in Δ*ssa* mutants. A: Cell extracts prepared from wt, Δ*ssaI*, Δ*ssaII*, and Δ*ssaI/II* strains grown in YPD at 30°C (lanes 1–4) or at 35°C (lanes 5–8). B: Cell extracts prepared from wt, Δ*ssaII*, and Δ*ssaII* cells carrying a single copy plasmid containing the *SSAII* gene under the control of its endogenous promoter (Δ*ssaII*+*pSSAII*). C: Cell extracts prepared from Δ*ssaI* and Δ*ssaI* cells carrying a single copy plasmid containing the *SSAII* gene under the control of its endogenous promoter (Δ*ssaI*+*pSSAII*). Western blot analyses were done using anti-Ape1p antibodies, or anti-hexokinase antibodies. The hexokinase control was used as an internal control.

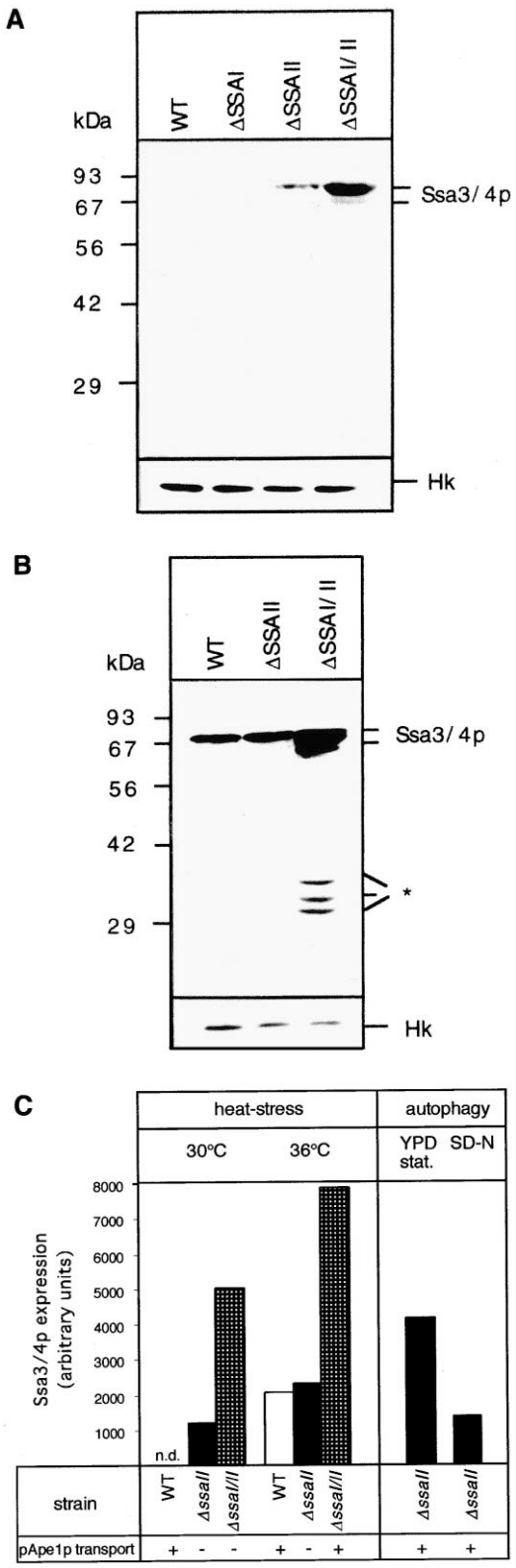

Fig. 2. Expression of Ssa3/4p in Δ*ssa* mutants. A: Cell extracts were prepared from wt, Δ*ssaI*, Δ*ssaII*, and Δ*ssaI/II* strains grown in YPD at 30°C. B: Cell extracts were prepared from wt, Δ*ssaII*, and Δ*ssaI/II* strains grown at an elevated temperature of 35°C. Western blot analyses were done using anti-Ssa3/4p antibodies or anti-hexokinase antibodies. *Degradation products of Ssa3/4p. C: Quantification of Ssa3/4p expression under growth conditions which bypass the Ssa1/2p requirement for pApe1p transport (n.d., not detectable).

missing Ssa1p (Fig. 1A, lane 2) or Ssa2p (Fig. 1A, lane 3) an accumulation of the 61 kDa precursor form (pApe1p) was observed. In mutants missing both chaperones (Fig. 1A, lane 4), no increase in the accumulation of the precursor was seen compared with the single deletion strains.

Expression of the Ssa2p from a single copy plasmid, containing the *SSAII* gene under its own promoter, is able to rescue the mutant phenotype in the Δ*ssaII* strain (Fig. 1B, compare lanes 1 and 3) as well as in the Δ*ssaI* strain (Fig. 1C, compare lanes 1 and 3). Vice versa Ssa1p rescues the mutant phenotype in the Δ*ssaII* strain (Fig. 1C, compare lanes 4 and 6) as well as in the Δ*ssaI* strain (data not shown), clearly demonstrating overlapping functions of Ssa1p and Ssa2p.

The loss of one or more members of the Ssap family can be compensated by overexpressing other family members [15]. Western blot analysis of cell extracts from wt and mutant strains with an antibody that recognizes Ssa3p and Ssa4p shows under steady-state conditions an overexpression of Ssa3/4p, in the Δ*ssaII* strain (Fig. 2A, lane 3) and to a higher degree in the Δ*ssaI/II* strain (Fig. 2A, lane 4). As in unstressed cells about 30% of the Ssa proteins are Ssa1p (the other 70% are Ssa2p), we barely observed an overexpression of Ssa3/4p in the Δ*ssaI* mutant (Fig. 2A, lane 2). Ssa3p and Ssa4p apparently compensate for the loss of Ssa1/2p function in the transport of pApe1p.

This conclusion is supported by an experiment in which wt yeast cells and *SSA* deletion mutants were grown at 30°C and shifted to an elevated temperature of 35°C for 1 h (Fig. 1A, lanes 5–8). In the Δ*ssaI/II* strain no accumulation of the precursor of Ape1p was seen (Fig. 1A, lane 8). Under these heat shock conditions Ssa3/4p are overexpressed in the Δ*ssaII* mutant (Fig. 2B, lane 2). However, Ssa3/4p overexpression is higher in the Δ*ssaI/II* strain (Fig. 2B, lane 3). These results suggest that under stress conditions, induced either by deletion of constitutively expressed Ssa proteins or by an elevated temperature, Ssa3p and Ssa4p are expressed at levels which can fully complement lack of Ssa1p and Ssa2p function in the transport of pApe1p into the vacuole.

mApe1p and pApe1p expression levels were found to be reduced at 30°C in Δ*ssa* strains (Fig. 1A). Ape1p expression is upregulated under heat stress at 35°C (Fig. 1A), which is known to elevate the expression levels of several vacuolar proteins [36]. At 35°C Ape1p expression increased in Δ*ssa* strains to wt expression levels and therefore we assume that reduced expression levels of Ape1p at 30°C in Δ*ssa* strains might be an indirect effect of Ssap deficiency. Deletion of the vacuolar proteinase A in the Δ*ssaI* strain did not increase

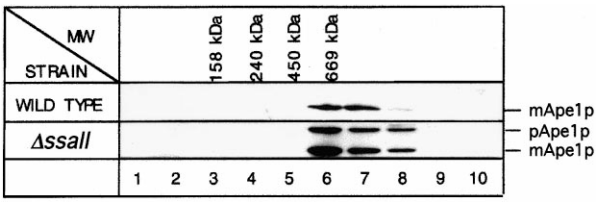

Fig. 3. The accumulated precursor of Ape1p forms a dodecamer. Cell extracts were prepared from wt and Δ*ssaII* cells. Proteins were separated according to their molecular weight on a glycerol density gradient, precipitated, separated by SDS–PAGE and immunoblotted with an antibody against Ape1p. Molecular weight standards indicated are aldolase (158 kDa), catalase (240 kDa), ferritin (450 kDa) and thyroglobulin (669 kDa). pApe1p: precursor Ape1p; mApe1p: mature form of Ape1p; MW: molecular weight.

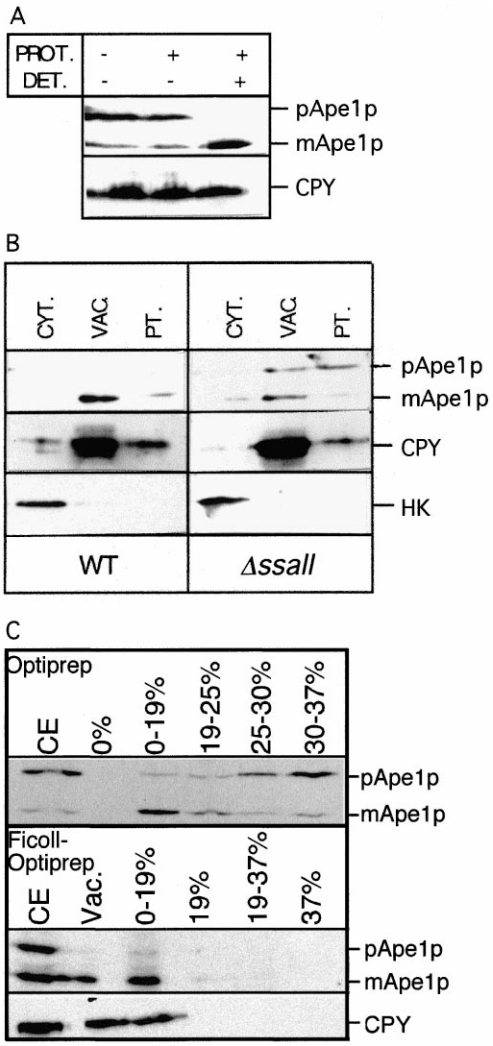

Fig. 4. The precursor of Ape1p accumulates in a prevacuolar compartment. A: Δ*ssaII* cells were spheroplasted, differentially lysed and protease-treated with proteinase K (PROT.) in the absence or presence of Triton X-100 (DET.). Western blot analysis was done with an antibody against Ape1p or CPY as a vacuolar marker. B: Wild-type or Δ*ssaII* cells were spheroplasted, differentially lysed and fractionated on a Ficoll gradient. The top fraction of the gradient containing cytoplasm (CYT.), a fraction containing vacuoles (VAC.) and the pellet of the gradient containing unlysed spheroplasts and prevacuolar vesicles (PT.) were collected. C: Upper panel: Optiprep gradient of Δ*ssaII* cell lysate (CE) and the interphases of the gradient analyzed by anti-Ape1p Western blot. Lower panel: Δ*ssaII* cell lysate (CE) and vacuolar fraction (Vac.) of the Ficoll gradient and the Optiprep gradient centrifugation of the vacuolar Ficoll gradient fraction. Western blot analyses were done for Ape1p or for the vacuolar marker CPY and hexokinase as a cytoplasmic marker.

the amount of Ape1p (not shown), excluding increased vacuolar turnover of Ape1p due to misfolding. We tested for direct interaction between Ssa1/2p and pApe1p by chemical crosslinking, purification of crosslinked complexes by anti-Ssa1/2p immunoaffinity columns followed by Western blot analysis, but we were not able to identify crosslinked products. This negative result does not exclude the possibility of a pApe1p–Ssap interaction prior to packaging of pApe1p into Cvt vesicles (see also Section 3.2).

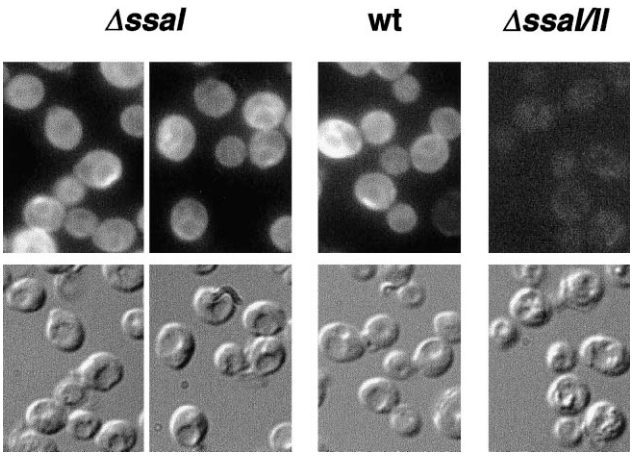

Fig. 5. Subcellular localization of Ssa1/2p. Upper panel: Immunolocalization of Ssa2p in Δ*ssa1* cells, of Ssa1/2p in wt cells. Cells deficient in both proteins served as anti-Ssa1/2p antibody control. Lower panel: Nomarski images of the cells.

3.2. Aminopeptidase 1 accumulates in the Δssa mutants in its dodecameric form in a prevacuolar compartment

Ape1p is synthesized as a monomer that rapidly oligomerizes into a pre-dodecamer [34]. To determine the oligomerization state of the accumulated precursor, seen in the Δ*ssa* mutants, protein extracts from the wt and the Δ*ssaII* strain grown to mid-exponential phase were prepared and proteins were separated according to their molecular weight on glycerol density gradients. The gradients were fractionated, the proteins in each fraction were TCA-precipitated and separated by SDS–PAGE, and Western blot analysis was performed using anti-Ape1p antibodies (Fig. 3). The mature dodecamer in the wt and the Δ*ssaII* strains migrated to a density corresponding to a molecular weight of about 600 kDa. In the absence of Ssa2p the precursor accumulated in the same fractions as the mApe1p (Fig. 3) suggesting that the precursor was already assembled in its dodecameric form of about 730 kDa.

To determine whether the pApe1p accumulates inside a membrane-surrounded compartment or in the cytoplasm [37], spheroplasts of the Δ*ssaII* strain were differentially lysed to disrupt the plasma membrane, but allowing the majority of vacuoles and Cvt vesicles to remain intact [27,29]. Protease protection experiments with these lysed spheroplasts indicated that the accumulating precursor in the Δ*ssaII* mutant is protease-protected unless detergent was added, suggesting that it is surrounded by a membrane (Fig. 4A). CPY, a resident vacuolar protein, is not degraded under those conditions.

To investigate whether the precursor accumulates within the vacuole or Cvt vesicles, subcellular fractionation experiments using discontinuous Optiprep and Ficoll gradients were performed [37]. On Ficoll gradients mApe1p was found in the vacuolar fraction (0/4% Ficoll) of the gradient in wt cell lysates as well as in Δ*ssaII* cell lysates, together with the vacuolar CPY. pApe1p of the Δ*ssaII* strain was found in the pellet fraction of the gradient, which is in agreement with data in the literature (Fig. 4B). We also detected pApe1p in the vacuolar fraction in the Δ*ssaII* strain (Fig. 4B) and to verify this cofractionation we performed Optiprep gradient centrifugations (Fig. 4C, upper panel). The 0–19% interphase contained the vacuoles and the 30–37% Optiprep interphase contained the Cvt vesicles. No pellet was formed during the centrifugation. This is also in accordance with previous data, where mApe1p was detected in the 0–19% interphase containing the vacuoles and pApe1p in the 30–37% interphase [37]. This demonstrates that pApe1p accumulates in a prevacuolar compartment in the Δ*ssaII* strain. Trace amounts of pApe1p corresponding to 5% of the total were found in the 0–19% interphase. To verify whether this fraction is associated with the vacuole we also loaded the 0–4% Ficoll interface, containing vacuoles and mApe1p as well as minor amounts of pApe1p on top of an Optiprep gradient [37]. mApe1p and pApe1p were not separated on the Optiprep gradient demonstrating association of 5% pApe1p with the vacuolar fraction (Fig. 4C, lower panel).

3.3. Ssa1/2p are localized to the vacuolar membrane in vivo

The function of Ssaps in pApe1p transport could lead to a concentration of Ssaps at the vacuolar membrane. To test this we performed immunofluorescence microscopy of the strains with an anti-Ssa1/2p antiserum (Fig. 5). Vacuolar membranes of wt cells showed a prominent labeling of the vacuolar membrane exceeding the expected strong cytoplasmic signal, whereas Δ*ssaI/II* cells did not show any membrane labeling. Ssa3/4p can functionally replace Ssa1/2p for pApe1p import. Therefore we wanted to know whether we could detect a similar vacuolar staining for Ssa3/4p. When probing cells deleted for *SSAI/SSAII* with antiserum recognizing Ssa3/4p, we noticed a much weaker overall immunofluorescence signal than in the case of Ssa1/2p. This reflected the much lower expression level of Ssa3/4p. Occasionally, but not in all cells, we observed a staining of the vacuolar membrane that was elevated over the cytoplasmic stain (data not shown). This may reflect limitations of the detection procedure or may indicate the failure of Ssa3/4p to get enriched at the vacuolar membrane well above the cytoplasmic concentration. However, Ssa2p and/or Ssa1p clearly accumulate at the vacuolar membrane in situ. We do not know on which face(s) of the vacuolar membrane Ssa1/2p are localized. Attempts to resolve this question by immunoelectron microscopy did not yield clear results (data not shown).

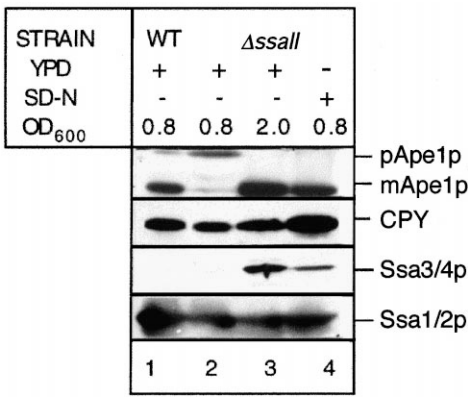

Fig. 6. Autophagocytosis can overcome the effect of Ssap deletion. Cell extracts were prepared from wt (lane 1) and Δ*ssaII* strains grown in YPD to an OD_{600} of 0.8 (lane 2), from Δ*ssaII* strains grown in YPD to an OD_{600} of 2.0 (lane 3) or grown in nitrogen starvation medium (SD-N) to an OD_{600} of 0.8 (lane 4). The proteins were separated by SDS–PAGE and western blotting was done using antibodies against Ape1p, CPY, Ssa1/2p and Ssa3/4p.

3.4. Autophagocytosis can overcome the effect of Ssap deletion

pApe1p has been detected in small Cvt vesicles as well as large autophagocytosis-like vesicles formed under starvation conditions. Could it be that the induction of autophagy by starvation can overcome the Ssap requirement of the pApe1p targeting? Ssa3/4p expression levels are reduced under starvation conditions [15]. The Ssa3/4p expression level in Δ*ssaII* under nitrogen starvation is as high as in the Δ*ssaII* strain at 30°C, which is insufficient to overcome the Ssa1/2p requirement for pApe1p transport (Fig. 6, lane 3, Fig. 2B, lane 3 and Fig. 2C). Also under starvation conditions after growth in rich medium Ssa3/4p expression levels are lower than in Δ*ssaI/II* at 30°C, where Ssa3/4p expression levels do not restore pApe1p transport to the vacuole (Fig. 2A, lane 4, Fig. 2B, lane 3, Fig. 1, lanes 4 and 8). pApe1p transport under starvation conditions would therefore occur independently of Ssa3/4p.

Cell extracts were prepared from wt (Fig. 6, lane 1) and Δ*ssaII* strains grown in rich medium to mid-exponential phase (Fig. 6, lane 2). To induce autophagocytosis Δ*ssaII* cells were grown to stationary phase (Fig. 6, lane 3) or grown in nitrogen starvation medium to mid-exponential phase (Fig. 6, lane 4). Under both starvation conditions no pApe1p accumulation was observed (Fig. 6, compare lanes 3 and 4 with lane 1), suggesting that autophagocytosis can overcome the Ssap requirement and that the Ssaps are specifically required in the Cvt pathway of pApe1p transport and not in other pathways as autophagocytosis.

4. Discussion

Using Δ*ssa* strains we found that cytoplasmic Hsp70 proteins are involved in the transport of pApe1p from the cytoplasm into the vacuole in yeast. As already known from other Ssap-dependent processes members of the Ssa protein family displayed overlapping functions and the overall amount of Ssa proteins is responsible for the severity of an observed phenotype. The accumulated precursor pApe1p forms a dodecameric complex that can be found inside a vesicle. The Δ*ssa* mutants behave like some Cvt and autophagocytosis mutants. As far as we know, it has never been reported that Cvt or autophagocytosis mutants are allelic with Δ*ssa* mutants.

In the Cvt model suggested by Kim and co-workers [34] the precursor obtains its dodecameric form already in the cytoplasm and is then taken up into an autophagosome-like vesicle which is transported into the vacuole by macroautophagocytosis. Inside the vacuole the vesicle is degraded and the maturation of pApe1p takes place. In this model Ssa proteins could target, according to their known functions, pApe1p precursor to a forming autophagocytotic vesicle, where they might also assist in the assembly of the pre-dodecamer, which is finally packed in these vesicles.

However, the data presented in this article suggest an alternative view. If the cell is depleted of cytoplasmic Hsp70s pApe1p accumulates in autophagosome-like Cvt vesicles. The transport and uptake of these vesicles into the vacuole is the rate-limiting step in pApe1p transport along the Cvt pathway in wt cells, because pApe1p can only be found in cytoplasmic Cvt vesicles under steady state and identification of other transport intermediates requires mutations of the precursor pApe1p itself or of the vacuolar hydrolases which degrade the vacuolar autophagic bodies [29]. The precursor accumulates in these vesicles in the *ssa* mutants. Thus Ssaps seem to be required for the fusion of Cvt vesicles with the vacuole. The mechanism of Cvt vesicle to vacuole fusion is not known. Studies on the homotypic vacuole fusion reaction have shown that formation of multimeric NSF, SNARE-containing protein complexes on the membranes required for docking and fusion, is supported by chaperones [38]. One may therefore speculate that Ssa1/2p are involved in the formation of such protein complexes required for Cvt vesicle to vacuole fusion. Vam3p is a vacuolar t-SNARE required for vacuolar membrane fusion with structures from the secretory pathway as well as the autophagocytotic pathway [39]. This indicates that the Cvt vesicle to vacuole membrane fusion reaction might use the same mechanisms as the homotypic vacuolar membrane fusion reaction. However, no Hsp70 involvement has been demonstrated in these reactions so far and the vacuolar CPY is found in its mature form in the vacuole of Ssap mutants. Formation of large protein complexes specific for autophagosome to vacuole fusion has also been described. Apg proteins form protein complexes required for autophagocytosis in yeast and mammals [40,41]. Apg12p and Apg5p are covalently linked by Apg10p. Apg10p is homologous to the E1 enzymes of the ubiquitin system. The Apg12p/Apg5p complex interacts with Apg16p to form a multimeric protein complex, but their role in the fusion process is not known [42]. Our results seem to rule out a participation of Ssaps in the fusion of autophagocytic vesicles with the vacuole.

The localization of the Ssa1/2p on the vacuolar membrane supports a function of these proteins in the Cvt vesicle to vacuole fusion reaction. Establishing an in vitro system for Cvt vesicle to vacuole membrane fusion will allow us to study the function of Ssa1/2p in the Cvt vesicle to vacuole fusion reaction.

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