The Heat Shock Protein Ssa2p Is Required for Import of Fructose-1,6-Bisphosphatase into Vid Vesicles

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Abstract. Fructose-1,6-bisphosphatase (FBPase) is targeted to the vacuole for degradation when *Saccharomyces cerevisiae* are shifted from low to high glucose. Before vacuolar import, however, FBPase is sequestered inside a novel type of vesicle, the vacuole import and degradation (Vid) vesicles. Here, we reconstitute import of FBPase into isolated Vid vesicles. FBPase sequestration into Vid vesicles required ATP and cytosol, but was inhibited if ATP binding proteins were depleted from the cytosol. The heat shock protein Ssa2p was identified as one of the ATP binding proteins involved in FBPase import. A Δ*ssa2* strain exhibited a significant decrease in the rate of FBPase degradation in vivo as compared with Δ*ssa1*, Δ*ssa3*, or Δ*ssa4* strains. Likewise, in vitro import was impaired for the Δ*ssa2* strain, but not for the other Δ*ssa* strains. The cytosol was identified as the site of the Δ*ssa2* defect; Δ*ssa2* cytosol did not stimulate FBPase import into import competent Vid vesicles, but wild-type cytosol supported FBPase import into competent Δ*ssa2* vesicles. The addition of purified recombinant Ssa2p stimulated FBPase import into Δ*ssa2* Vid vesicles, providing Δ*ssa2* cytosol was present. Thus, Ssa2p, as well as other undefined cytosolic proteins are required for the import of FBPase into vesicles.

Key words: molecular chaperones • vesicle trafficking • • protein degradation • Ssa2p yeast vacuole

Introduction

The vacuole of the yeast *Saccharomyces cerevisiae* is homologous to the lysosome of higher eukaryotes (Klionsky et al., 1990; Jones, 1991; Raymond et al., 1992). This organelle contains a variety of proteolytic enzymes that are important in degrading normal proteins, overexpressed proteins, and some abnormal proteins (Klionsky et al., 1990; Jones, 1991; Raymond et al., 1992). Proteins are targeted to the vacuole by one of several mechanisms. The most studied example is the sorting of the vacuole lumenal protein carboxypeptidase Y (CPY)[1] from the late secretory pathway (Klionsky et al., 1990; Jones, 1991; Raymond et al., 1992). CPY is synthesized, translocated into the ER, and transported to the Golgi body, where it is sorted from the late Golgi body by the CPY receptor, and is delivered to the vacuole through the late endosome/prevacuolar compartment (Rothman and Stevens, 1986; Marcusson et al., 1994; Cooper and Stevens, 1996). To date, >40 genes involved in this process, vacuolar protein sorting (*VPS*) genes, have been identified. Examples include the *VPS15* gene that encodes a protein kinase that recruits the phosphatidylinositol 3-kinase protein Vps34p to the membranes (Schu et al., 1993; Stack et al., 1993), likewise, the *VPS45* and *VPS27* genes, which are important for CPY trafficking into and out of the prevacuolar compartment, respectively (Conibear and Stevens, 1995; Piper et al., 1995). Finally, a novel pathway for sorting vacuole membrane proteins, such as alkaline phosphatase has been identified. This pathway is dependent on the adaptor protein, AP3 (Cowles et al., 1997; Piper et al., 1997).

Other vacuole resident proteins, such as aminopeptidase 1 (AP1) and α–mannosidase, are targeted from the cytoplasm to the vacuole independent of the secretory pathway (Yoshihisa and Anraku, 1990; Harding et al., 1995; Scott and Klionsky, 1998). AP1 targeting to the vacuole, for example, occurs by two routes (Baba et al., 1997). Under normal growth conditions, AP1 is delivered to the vacuole by CVT (cytoplasm to vacuole targeting) vesicles that are 140–160 nm in diameter. When cells are starved of nitrogen, however, AP1 is delivered to the vacuole by the macroautophagy pathway. Autophagosomes of 300–900 nm with double membranes are formed in the cytoplasm. After fusion with the vacuole, the outer membrane becomes part of the vacuolar membrane, and the intact autophago-

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[1]*Abbreviations used in this paper:* AP1, aminopeptidase 1; CPY, carboxypeptidase Y; FBPase, fructose-1,6-bisphosphatase; hsc, heat shock cognate protein; Hsp, heat shock proteins; Vid, vacuole import and degradation; VPS, vacuolar protein sorting; WT, wild-type.

somes are delivered to the lumen of the vacuole (Baba et al., 1997).

A number of genes have been shown to play an important role in the macroautophagy process. For example, a novel ubiquitin-like conjugation system has been identified in a nonselective macroautophagy pathway that is induced under nitrogen starvation. This pathway utilizes the COOH-terminal glycine residue of Apg12 conjugated to a lysine residue of Apg5. In addition, this nonubiquitin conjugation system is dependent upon Apg10, as well as Apg7, a ubiquitin E1-like enzyme (Mizushima et al., 1998). Other proteins involved in the macroautophagy pathway include Apg8p/Aut7p. This protein has been shown to be required for autophagosome formation (Kirisako et al., 1999) and is essential for macroautophagy in yeast (Lang et al., 1998; Kirisako et al., 1999). Likewise, in mammalian cells, the tumor suppressor protein, beclin-1, has recently been shown to play a role in the macroautophagy pathway (Liang et al., 1999).

Fructose-1,6-bisphosphatase (FBPase), the key regulatory enzyme in gluconeogenesis in *S. cerevisiae*, is induced when yeast cells are grown in medium containing poor carbon sources, such as pyruvate, acetate, and oleate. FBPase is rapidly inactivated when fresh glucose is added to glucose-starved yeast cells (Gancedo, 1971) and is subsequently targeted to the vacuole for degradation (Chiang and Schekman, 1991; Chiang et al., 1996). The redistribution of FBPase from the cytosol to the vacuole has been observed by immunofluorescence techniques, cell fractionation, and EM (Chiang and Schekman, 1991; Chiang et al., 1996). In addition, the glucose-induced targeting of FBPase to the vacuole has been reconstituted in vitro using permeabilized yeast cells. In this model system, the sequestered FBPase is localized to the vacuole, the final destination for FBPase degradation (Shieh and Chiang, 1998).

Recent evidence suggests that FBPase is imported into a novel type of vesicle during the degradation process (Hoffman and Chiang, 1996; Huang and Chiang, 1997). These vacuole import and degradation (Vid) vesicles are distinct from the ER, Golgi body, vacuole, endosomes, mitochondria, peroxisomes, COPI, or COPII transport vesicles, and can be purified to near homogeneity. Furthermore, kinetic studies indicate that FBPase targeting to the vesicles occurs before uptake by the vacuole (Huang and Chiang, 1997). Accordingly, the vesicle-mediated FBPase degradation pathway can now tentatively be divided into two steps. The first step is the targeting and sequestration of FBPase into Vid vesicles. The second step is the delivery of FBPase from the vesicles to the vacuole for degradation.

As the intermediate carriers in the FBPase degradation pathway, Vid vesicles are predicted to contain proteins participating in FBPase import, and also proteins mediating FBPase trafficking from the Vid vesicles to the vacuole. A number of proteins participate in the FBPase trafficking process, as determined by our genetic studies (Hoffman and Chiang, 1996). Along these lines, we have isolated several *vid* mutants that are defective in the glucose-induced degradation of FBPase. Some *vid* mutants accumulate FBPase in the cytosol, whereas others accumulate FBPase in the vesicles. However, at present, only one of the *VID* genes that plays a role in the trafficking of FBPase has been characterized. This gene, *VID24*, encodes a 41-kD protein that is induced by glucose and is localized to the Vid vesicles (Chiang and Chiang, 1998). FBPase accumulates in the vesicles in the *vid24-1* mutant, suggesting that Vid24p plays an important role in FBPase trafficking from the Vid vesicles to the vacuole.

In mammalian cells, lysosomal degradation of proteins after starvation requires the presence of a pentapeptide sequence (Chiang and Dice, 1988; Chiang et al., 1989; Terlecky et al., 1992), and is mediated by heat shock proteins (Hsp; Terlecky and Dice, 1993; Cuervo et al., 1994). The cytosolic heat shock cognate protein, hsc73, binds to proteins that are targeted for degradation, and as such plays an important role in the import of these proteins into the lumen of the lysosome. The hsc73 protein is also found inside the lumen of the lysosomal compartment and is necessary for uptake of proteins into the lysosomal lumen (Agarraberes et al., 1997; Cuervo et al., 1997). In a similar manner, members of the hsp70 family have been found to play a role in the import of proteins into the vacuole of yeast (Horst et al., 1999). For example, the depletion of cytosolic members of the hsp70 family resulted in a significant decrease in the uptake of proteins into isolated yeast vacuoles. As yet, it is unknown whether there are lumenal vacuolar chaperones that play a role in protein import, although data from other organelle systems suggests that this is highly probable.

To identify molecules that participate in the FBPase targeting into Vid vesicles, we developed an in vitro system using isolated Vid vesicles and cytosol. This system reconstitutes sequestration of FBPase into Vid vesicles and provides a functional assay to identify molecules required for the import process. Here, we show that FBPase import is ATP-dependent and requires the presence of cytosol. Furthermore, we have identified the Hsp Ssa2p as an essential cytosolic component in the FBPase import into the Vid vesicles.

Materials and Methods

Media and Yeast Strains

The yeast strains used in this study are listed in Table I. Yeast were cultured in YPD (a complete medium containing 10 g/liter of Bacto yeast extract, 20 g/liter of Bacto peptone, 20 g/liter dextrose) or in YPKG (10 g/liter Bacto yeast extract, 20 g/liter Bacto peptone, 10 g/liter potassium acetate, 5 g/liter dextrose). For some experiments, yeast were cultured in a synthetic minimal medium (SD) consisting of 6.7 g/liter yeast nitrogen base without amino acids, supplemented with 20 mg/liter uracil, 20 mg/liter tryptophan, 20 mg/liter histidine, 30 mg/liter leucine, 30 mg/liter lysine, and 20 g/liter dextrose.

Antibodies

Rabbit anti-FBPase, anti-PMA, antithiolase, anticytochrome C, and antienolase polyclonal antibody were generated by BabCo using purified proteins as antigens. Anti-Sec21p antibody was provided by Dr. C. Barlowe (Dartmouth Medical School, Hanover, NH) and anti-Pep12p was a gift from Dr. S. Emr (University of California at San Diego, La Jolla, CA). Anti-HA antibody was purchased from Boehringer and an anti-Vph1p antibody was purchased from Molecular Probes. Antibodies were used at 1:2,000 dilution for Sec21p and Pep12p and at 1:10,000 dilution for Pma1p, thiolase, Vph1p, cytochrome C, enolase, HA, and FBPase.

Table I. Yeast Strains Used in this Study

Strain	Genotype
HLY223	*Mata leu2-3, 112 his3 trp1-1 ura3-52 lys2-801*
HLY218	*Mata leu2-3, 112 his3 trp1-1 HA::VID24 lys2-801*
JN114	Matα *his3-11,15 leu2-3,112 ura3-52 Δtrp1 lys2 ssa1::HIS3*
JN115	Matα *his3-11,15 leu2-3,112 ura3-52 Δ trp1 lys2 ssa2::URA3*
MW127	Matα *his3-11,3-15 leu2-3,112 lys3 Δtrp1 ura3-52 ssa3*::TRP1
JN294	Matα *his3-11,3-15 leu2-3,112 ura3-52 Δtrp1 lys2 ssa4::LYS*
MW123	Matα *his3-11,15 leu2-3,112 lys3 Δtrp1 ura3-52 ssa1::HIS3 ssa2::LEU2*
MW328	Matα *his3-11,15 leu2-3,112 lys3 Δtrp1 ura3-52 ssa1::HIS3 ssa3::TRP*
HLY193	*Mata leu2-3, 112 his3 trp1 ura3-52 fbp::LEU2*
HLY361	Matα *his3-11,15 leu2-3,112 ura3-52 Δtrp1 lys2 ssa1::HIS3 fbp::LEU2*
HLY362	Matα *his3-11,15 leu2-3,112 ura3-52 Δ trp1 lys2 ssa2::URA3 fbp::LEU2*
HLY363	Matα *his3-11,15 leu2-3,112 lys3 Δtrp1 ura3-52 ssa3::TRP1 fbp::LEU2*
HLY364	Matα *his3-11,3-15 leu2-3,112 ura3-52 Δ trp1 lys2 ssa4::LYS fbp::LEU2*

Preparation of Purified Proteins

FBPase was purified according to the procedure described by Rittenhouse et al. (1986), except that the P11 column was omitted. FBPase was the major protein band (80% of the total protein) on the Coomassie blue stained gels. FBPase from these preparations were 5–10 mg/ml as determined by BioRad D_c protein assay kit. The enzyme was stored at −70°C in small aliquots.

ATP binding proteins were isolated using ATP agarose (Sigma-Aldrich) immobilized on cross-linked 4% beaded agarose. For some experiments, 100 μl of cytosolic proteins (~1.5 mg) from wild-type (WT) cells were added directly to an ATP agarose column. The flow-through material (cytosol depleted of ATP binding proteins) was collected and saved for further use in the in vitro FBPase import experiments. ATP binding proteins were eluted with 100 μl of import buffer containing 10 mM ATP, and were likewise used in the in vitro assay. As a control, 100 μl of cytosol was passed over an agarose column lacking cross-linked ATP. For other experiments, ATP binding proteins were collected from cytosol that was partially purified using DEAE chromatography, a variation of a protocol used to purify Ssa proteins (Chirico et al., 1988). WT cytosol (100 mg) was passed over a DEAE column and eluted with buffer containing 300 mM KOAc. Fractions were collected and examined by SDS-PAGE and Western blots. Fractions containing the highest concentrations of Ssa proteins were pooled and salt exchanged to FBPase import buffer using a G-25 column. The material was applied to the ATP column as described above. The ATP binding proteins were eluted with buffer containing 10 mM ATP. Fractions containing the highest concentration of Ssa proteins (0.5 mg/ml) were used for the in vitro assay.

pYES2.1 TOPO plasmids (Invitrogen) were used to express either Ssa1p or Ssa2p. The *SSA1* and *SSA2* genes were linked to a His-tag coding sequence and the plasmid constructs were transformed into yeast strain HLY223. The cells were grown in SD to an OD_{600} = 1 and then transferred to YPGal medium (1% yeast extract, 2% peptone, 2% galactose, 0.1% raffinose) and grown to an OD_{600} approximating 6.0. Cells were harvested, resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCL, pH 7.9), and lysed. The yeast lysate containing soluble recombinant proteins was loaded onto His-bind Ni^{2+} Sepharose resin (Novagen). Ni^{2+} Sepharose with bound target proteins was washed with three bed volumes wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCL, pH 7.9) to elute nonspecific bound contaminants. 6X-His–tag protein was eluted from the Ni^{2+} Sepharose with elute buffer (50–500 mM imidazole, 250 mM NaCl, 10 mM Tris-HCl, pH 7.9) in 5-ml gradients and collected in 1.0-ml fractions. Collected fractions were examined via SDS-PAGE, Western Blot, and BioRad protein assay to identify the 6X-His–tag protein and determine concentrations. Protein aliquots (~0.5 mg/ml) were stored at −70°C before their use.

Differential Centrifugation

Yeast cells were grown in 10 ml of YPKG for 2 d at 30°C in an environmental shaker (300 rpm). Cells (OD_{600} = 40) were harvested by centrifugation at 500 *g* for 5 min and then shifted to YPD for 20 min at 30°C. At the end of incubation, 10 mM NaN_3 was added to the culture and yeast cells were collected by centrifugation. Cells were resuspended in 1 cell volume of import buffer (0.4 M sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes-KOH, pH 6.6) supplemented with 200 μg/ml phenylmethylsufonyl fluoride and 2 vol of ice-chilled glass beads (0.45–0.50 mm). The mixture was vortexed at the highest speed for 1 min and then chilled on ice for 5 min. This procedure was repeated five times or until >90% of the cells were disrupted as determined by light microscopy. Cell debris was removed by centrifugation at 700 *g* for 10 min. The low speed supernatant was then centrifuged at 13,000 *g* for 20 min in a desktop centrifuge (American Scientific Products). The 13,000 *g* supernatant was further centrifuged at 100,000 *g* for 2 h using a TLA 100.2 rotor and Beckman ultracentrifuge. The 100,000 *g* supernatant was further fractionated by centrifugation at 200,000 *g* for 2 h using the TLA 100.2 rotor. Proteins from the pellet fractions were resuspended in 100 μl SDS-loading buffer, whereas the 200,000 *g* supernatant was first precipitated with 10% TCA and then resuspended in 100 μl of SDS loading buffer. Proteins (15 μl) were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes for immunoblotting. The presence of FBPase and the other marker proteins mentioned above were detected using the ECL immunoblotting procedure.

Preparation of Vid Vesicles and Cytosol

Vid vesicle material was obtained using the differential centrifugation techniques described above, with minor modifications. Yeast cells were grown in 250 ml flasks for 2 d in YPKG media to a final concentration of OD_{600} = 600. Cells were pelleted and resuspended in YPD media and incubated for an additional 20 min at 30°C. The cells were then chilled and pelleted at 3,000 *g* at 4°C. The cell pellet was resuspended in import buffer and subjected to the differential centrifugation steps as described above. After centrifugation, the pellets were resuspended in import buffer and protein concentrations were determined by BioRad protein assays. Pellet and supernatant aliquots were frozen at −70°C until use in the import assay.

The FBPase Import Assay

The FBPase import assay was based upon a modification of a technique developed by Shieh and Chiang (1998). In a typical experiment, the reaction mixture (100 μl) contained 5 μg of vesicle material, 50 μg of cytosolic proteins, 11 μg of FBPase, FBPase import buffer, and an ATP regenerating system (0.5 mM ATP, 0.2 mg/ml creatine phosphokinase, and 40 mM creatine phosphate). The reaction mixture was incubated at 30°C for 20 min to allow for FBPase import. Proteinase K (0.8 mg/ml) was added and the mixture was incubated at room temperature for 15 min. The reaction was terminated by the addition of 1 ml of 15% wt/vol trichloroacetic acid (TCA) solution. Samples were centrifuged at 13,000 *g* for 10 min at 4°C, after which the precipitate was washed once with ice-cold acetone and resuspended in 200 μl of SDS-loading buffer. Proteins (15 μl) were resolved on SDS-PAGE and examined by Western blot analysis. To examine whether FBPase was sequestered inside a membranous compartment, 0.8 mg/ml proteinase K was added to the reactions in the absence or presence of 2% Triton X-100. The import of FBPase was defined as the fraction of the full-length 38 kD FBPase that was protected from proteinase K digestion in the absence of Triton X-100, but was sensitive to proteinase K in the presence of Triton X-100.

Results

Isolation of Vid Vesicles

We have developed a number of model systems to study the trafficking and degradation of FBPase (Chiang and Schekman, 1991; Chiang et al., 1996; Hoffman and Chiang, 1996; Huang and Chiang, 1997). Using these systems, we determined that the FBPase degradation pathway consists of at least two steps. The first step involves the movement of FBPase into Vid vesicles, while in the second step, Vid vesicles are targeted to the vacuole, where FBPase is degraded. Recently, we used semi-intact cells to reconstitute the trafficking of FBPase to the vacuole (Shieh and Chiang, 1998). By this assay, FBPase was detected in the vacuole as shown by immunofluorescence microscopy. However, it was difficult to assess whether or not FBPase first localized to Vid vesicles before entry into the vacuole. There-

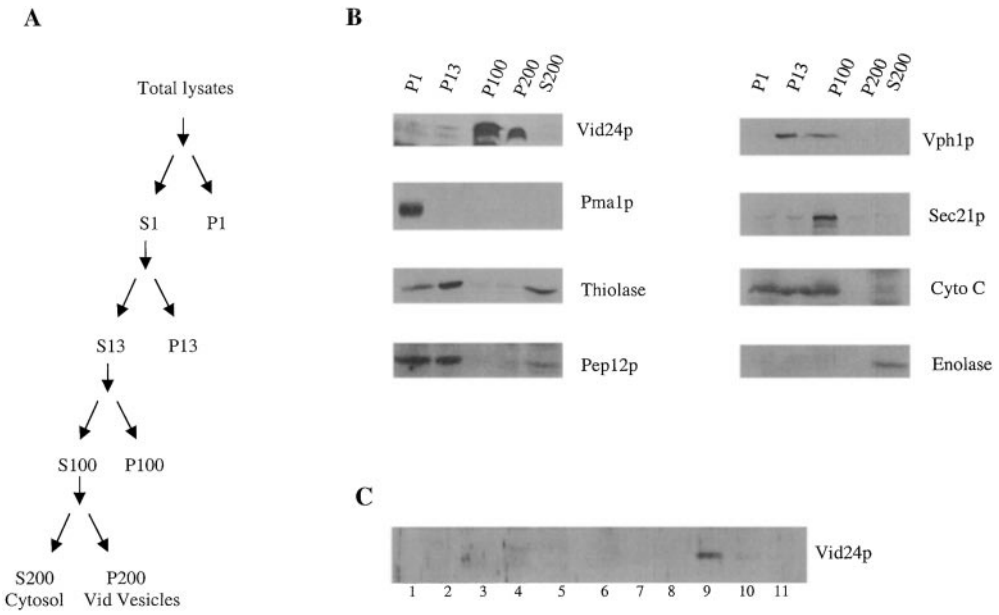

Figure 1. Isolation of Vid vesicles. A WT yeast strain expressing HA-Vid24p was grown in YPKG media and then shifted to glucose containing YPD media for 20 min. A, The cells were harvested and fractionated via a series of differential centrifugation steps. Cells were initially subjected to a 1,000-*g* spin to remove unbroken cells and nuclei (P1). The resultant supernatant (S1) was subjected to a series of additional 13,000-*g* and 100,000-*g* centrifugation steps ending with a final 200,000-*g* spin from which cytosol (S200) and vesicles (P200) were obtained. B, The distribution of organelles in differential centrifugation fractions was determined by immunoblotting with organelle specific antibodies: Vid24p for Vid vesicles (40 kD), Pma1p for the plasma membrane (106 kD), 3-oxacyl CoA thiolase for peroxisomes (45 kD), Pep12p for endosomes (35 kD), Vph1p for vacuoles (100 kD), Sec21p for COPI vesicles (105 kD), cytochrome C for mitochondria (13 kD), and enolase for cytosol (47 kD). C, The presence of Vid vesicles in the P200 fraction was verified by sucrose gradient density centrifugation. The P200 fraction was resuspended in buffer and separated using a 20–50% sucrose gradient. Fractions were collected and examined for the presence of HA-tagged Vid24p.

fore, to address this question, we attempted to develop a more refined in vitro assay in which we can reproduce the targeting of FBPase into Vid vesicles.

Vid vesicles can be purified to near homogeneity via the use of a combination of differential centrifugation, sizing chromatography, and sucrose gradient centrifugation steps (Huang and Chiang, 1997). In our initial studies, we attempted to utilize these vesicles as part of an in vitro system. Unfortunately, we were unable to reconstitute the import of FBPase. Although there are many possible explanations, we suspect that the number of steps and the time required for the purification process was a major problem, particularly owing to the fragile nature of Vid vesicles. Therefore, we attempted to develop a more rapid purification protocol. For these experiments, yeast were grown for two days in YPKG media to induce the production of FBPase. The cells were then shifted to growth in YPD media for 20 min, a procedure that resulted in the maximum FBPase import in semi-intact cells (Shieh and Chiang, 1998). The cells were harvested and subjected to a series of differential centrifugation steps to separate Vid vesicles from other cellular organelles and membranes (Fig. 1 A).

To identify fractions that were enriched for Vid vesicles, we screened for the presence of organelle marker proteins (Fig. 1 B). Vid24p was used as a marker protein to identify Vid vesicles, since this protein has been shown previously to associate with these structures (Chiang and Chiang, 1998). The majority of Vid24p was found in the 100,000-*g* and 200,000-*g* pellets as determined by Western blot analysis. Therefore, both of these fractions contain Vid vesicles. Note, however, that the 200,000-*g* pellet contained significantly less total protein as determined by BioRad protein assay (0.3 mg for the 200,000-*g* pellet, relative to 2 mg for the 100,000-*g* pellet). Therefore, based upon these data, we believe that the 200,000-*g* pellet is more highly enriched in Vid vesicles.

A number of marker proteins were utilized to determine whether any other organelles fractionated with Vid vesicles. The plasma membrane ATPase was detected with an anti-Pma1p antibody, whereas an antibody directed against 3-oxoacyl CoA thiolase was used to detect peroxisomes. Pep12p, a syntaxin homologue, was used to identify endosomes (Becherer et al., 1996). An antibody against the 100-kD subunit of the vacuolar proton ATPase (Vph1p) was used to detect the vacuoles. COPI vesicles, which are involved in the retrograde transport of proteins from Golgi to ER in yeast, were detected with antibodies against Sec21p (Bednarek et al., 1995). A cytochrome C antibody was used to detect mitochondria, whereas cytosolic fractions were identified with an antienolase antibody. As expected, enolase was only found in the 200,000-*g* cytosol. Likewise, a small amount of thiolase and cytochrome C was also found in this fraction. This most likely represents lumenal proteins from organelles that have been disrupted during the fractionation procedure. However, the majority of the marker proteins were found primarily in the 1,000-*g* (Pma1p), 13,000-*g* (thiolase, Pep12p, Vph1p), or 100,000-*g* (Sec21p) pellets, the location where these various organelle markers were expected to partition. Note that there were little, if any, of these proteins found in the 200,000-*g* pellet fraction.

To further demonstrate that the 200,000-*g* fraction contains Vid vesicles, a sucrose gradient fraction experiment was conducted. The 200,000-*g* pellet was resuspended in buffer and applied to a 20–50% sucrose gradient, which

was then centrifuged at 100,000 *g* for 16 h. Fractions were collected and analyzed for the location of Vid24p. As shown in Fig. 1 C, the majority of Vid24p was found in fractions 9 and 10. This is consistent with our previous report in which isolated Vid vesicles were observed to migrate to these same locations (Chiang and Chiang, 1998). Thus, this further supports our contention that the 200,000-*g* pellet is a source of highly enriched Vid vesicles.

In Vitro Import of FBPase into Vid Vesicles

The presence of Vid vesicles in the 100,000- and 200,000-*g* pellets left open the possibility that this material could be used as part of an in vitro FBPase import reaction mixture. To test whether the 100,000- and 200,000-*g* pellets were competent for FBPase import, the pellets were incubated in the presence of an import cocktail containing ATP and an ATP regenerating system. Note that the 100,000- and 200,000-*g* pellets and the cytosol used in these experiments were derived from a strain in which the endogenous *FBP1* gene had been deleted. Consequently, a known quantity of purified FBPase was also included in the reaction mixture. After a 20 min incubation period, proteinase K was added to degrade unprotected FBPase and the reaction was terminated by the addition of TCA. The 100,000-*g* pellet was not competent for FBPase import, as indicated by the absence of protected FBPase (Fig. 2 A). However, there was a significant amount of protected FBPase, if both the 100,000-*g* supernatant and pellet were included in the reaction. Surprisingly, the 100,000-*g* supernatant fraction was observed to protect FBPase from proteinase K degradation even in the absence of added 100,000-*g* pellet (Fig. 2 A). Thus, it appears that the 100,000-*g* supernatant contains all of the components that are necessary for FBPase import (e.g., Vid vesicles and cytosolic factors), and could potentially be used for the in vitro assay.

To more clearly define the role that cytosolic proteins play in the import of FBPase, we subjected the 100,000-*g* supernatant to a further 200,000-*g* fractionation step. After this additional purification step, neither the 200,000-*g* supernatant (cytosol) nor the 200,000-*g* pellet (Vid vesicles) protected FBPase from proteinase K digestion (Fig. 2 B). However, the combination of cytosol and Vid vesicle fractions did result in import, as indicated by the presence of protected FBPase. Interestingly, the 200,000-*g* cytosol could also drive import of FBPase into the 100,000-*g* pellet (Fig. 2 C). Therefore, it appears that both the 100,000- and 200,000-*g* pellets contain Vid vesicles that are competent for the in vitro import of FBPase, although the 200,000-*g* pellet appears to be more highly enriched in Vid vesicles. In contrast to the preceeding results, a minimal amount of FBPase import was observed when cytosol was combined with the 13,000-*g* pellet (Fig. 2 C), a fraction that is enriched in the vacuolar marker protein, Vph1p (Fig. 1 B). Although we suspect that there may be some direct import of FBPase into vacuoles, we could not reconstitute this using the crude 13,000-*g* fraction. Therefore, this FBPase import assay appears to be specific for fractions that are enriched in Vid vesicles.

The preceding data suggests that the 100,000- and 200,000-*g* pellets contain Vid vesicles that protect FBPase from proteinase K digestion. To further verify the presence

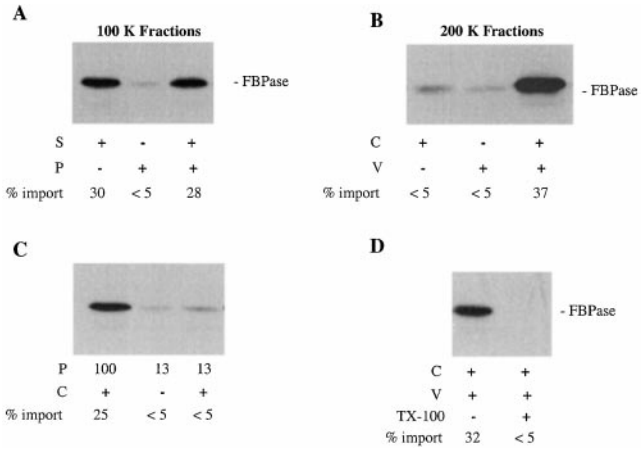

Figure 2. FBPase can be imported into isolated Vid vesicles. A, FBPase import was carried out using the import mixture described in Materials and Methods. Various 100,000-*g* supernatant (S) and pellet (P) fractions were added individually or in combination to identify the components necessary for FBPase import. The reaction was terminated by the addition of 0.8 mg/ml proteinase K and subsequent TCA precipitation. B, The FBPase import reaction was conducted as described above using the 200,000-*g* cytosol (C) and vesicle (V) fractions. C, The FBPase import reaction was conducted using the S200 cytosol and either the P100 or P13 pellets. D, The FBPase import reaction was conducted using the S200 cytosol and P200 vesicles. Triton X-100 was added to the import reaction before the addition of proteinase K to determine whether FBPase protection required intact membranes. The percentage of added FBPase that was protected from proteinase K digestion was calculated (% import).

of membrane-bound Vid vesicles in the 200,000-*g* pellet, we examined the effect that detergent had on proteinase K protection. Cytosol and Vid vesicles were mixed and incubated with FBPase as described above. Under control circumstances, ~20–40% of the exogenously added FBPase was imported in vitro. However, when 2% Triton X-100 was added to solubolize the membranes, almost all of the exogenously added FBPase was degraded after the addition of proteinase K (Fig. 2 D). This offers further support for the idea that the protected FBPase is inside a membrane sealed compartment, most likely the Vid vesicles, and that FBPase import depends upon the presence of intact Vid vesicles.

ATP and ATP Binding Proteins Play a Role in FBPase Import

In the absence of exogenously added ATP, there was little import of FBPase into Vid vesicles (Fig. 3 A). However, there was a significant increase in FBPase import after the addition of ATP. Thus, it appears that ATP-dependent processes are involved in the import of FBPase into the Vid vesicles. ATP could potentially play a number of roles in the import of FBPase into Vid vesicles. ATP could interact with proteins on the surface of Vid vesicle and thereby stimulate import. Alternatively, certain cytosolic proteins may be dependent upon the presence of ATP to elicit their function. To help elucidate the site of action of these processes, cytosol was passed over an ATP agarose column to deplete this material of ATP binding proteins.

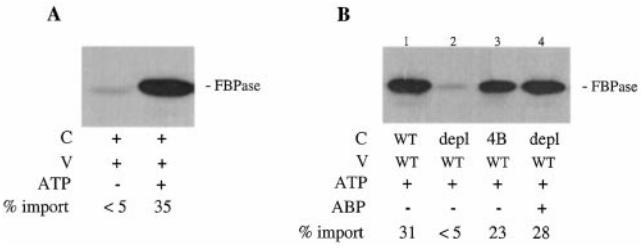

Figure 3. FBPase import into vesicles requires ATP and cytosol. A, The FBPase import reaction was conducted using the standard reaction mixture, but in the presence or absence of ATP. B, Cytosol was passed over an ATP column to deplete this material of ATP binding proteins. The depleted (depl) cytosol (50 μg) was then used in the FBPase import reaction. In lane 1, the complete reaction mixture was used, whereas in lane 2, depleted cytosol (depl) was used in the reaction mixture. As a control, cytosol was passed over a 4B column before use in the in vitro reaction (lane 3). ATP binding proteins (ABP) were eluted from the ATP agarose and 7 μg was added back to the reaction mixture to restore import (lane 4).

The depleted cytosol was then used in the in vitro assay. Cytosol that was passed over the ATP column had a greatly reduced ability to stimulate FBPase import into Vid vesicles (Fig. 3 B, lane 2). In contrast, the passage of cytosol over a 4B column had only a minor detrimental effect on FBPase import (Fig. 3 B, lane 3), suggesting that the above results are due to the binding of cytosolic proteins to ATP. Furthermore, FBPase import was restored if the ATP binding material was eluted from the ATP agarose column and added to the reaction mixture (Fig. 3 B, lane 4). Thus, this suggests that cytosolic ATP binding proteins play an important role in FBPase import.

SSA2 Is Required for FBPase Import

Molecular chaperones of the hsp70 family are ATPases that are known to play an important role in the import of proteins into various organelles, such as the ER (Chirico et al., 1988; Deshaies et al., 1988), the mitochondria (Hachiya et al., 1995), and the lysosome (Chiang et al., 1989; Cuervo and Dice, 1996; Hayes and Dice, 1996). Furthermore, these proteins are known to bind ATP and can be depleted from cytosolic fractions by ATP column chromatography (Chirico et al., 1988; Deshaies et al., 1988). Therefore, the yeast hsp70 chaperone family members (Ssa's) were considered to be potential candidates as the ATPases involved in FBPase import. In yeast, there are four Ssa isoforms. These include Ssa1p and Ssa2p, which are expressed constituitively, and Ssa3p and Ssa4p, which are heat-inducible and are not expressed under normal growth conditions (Werner-Washburne et al., 1988). To determine which Ssa proteins played a role in FBPase degradations, we used isogenic yeast strains with deletions of either *SSA1*, *SSA2*, *SSA3*, or *SSA4*. Each of these strains synthesized and accumulated FBPase after two days of growth in low glucose YPKG media cells (Fig. 4). However, there was a significant strain-related difference in FBPase degradation rates after incubation in glucose containing YPD media (see Fig. 4, G and H, for a quantitation of these results). For example, both the Δ*ssa3 and* Δ*ssa4* strains degraded FBPase

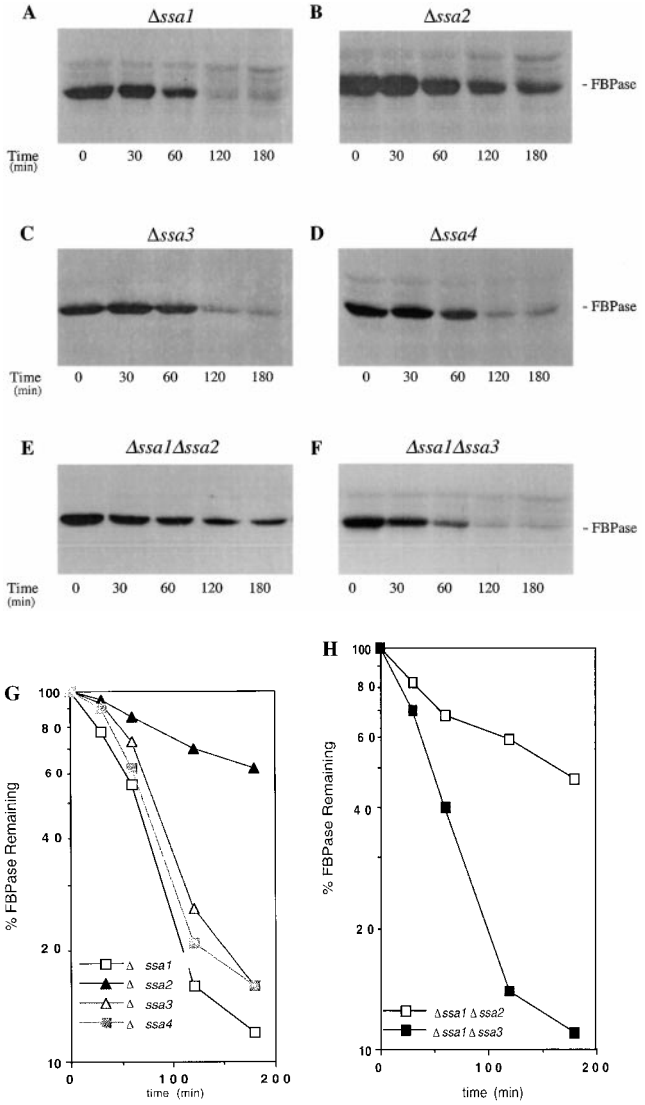

Figure 4. FBPase degradation is defective in Δ*ssa2* cells. Yeast strains containing deletions of various *SSA* genes were grown in YPKG media for 2 d to induce the production of FBPase and then shifted to YPD for various lengths of time. The cells were harvested and examined by SDS-PAGE and Western blot analysis using an anti-FBPase antibody. A, Δ*ssa1* strains. B, Δ*ssa2* strains. C, Δ*ssa3* strains. D, Δ*ssa4* strains. E, Δ*ssa1*Δ*ssa2* strains. F, Δ*ssa1*Δ*ssa3* strains. G, Quantitation of the kinetics of FBPase degradation in the Δ*ssa1*, Δ*ssa2*, Δ*ssa3*, *and* Δ*ssa4* strains. Values for the percentage of FBPase remaining at the various time points were determined relative to FBPase present at the initiation of the glucose shift (t = 0). H, The kinetics of FBPase degradation for the Δ*ssa1*Δ*ssa2* and Δ*ssa1*Δ*ssa3* strains was quantitated as described above.

with kinetics similar to WT strains; there was a significant decrease in FBPase levels by 60 min, and little detectable FBPase by 120 min (Fig. 4, C and D). This was as expected, since neither Ssa3p nor Ssa4p are constituitively expressed and, as such, should not play a role in the degradation of FBPase. In contrast, the Δ*ssa2* strain was defective in FBPase degradation, as indicated by the continued presence of FBPase, even at the 180-min time point (Fig. 4 B). Interestingly, the Δ*ssa1* strain did not appear to be de-

fective in FBPase degradation (Fig. 4 A), even though the *SSA1* gene is ~97% homologous to the *SSA2* gene (Boorstein et al., 1994). Thus, it appears that Ssa2p, but not Ssa1p, is required for the degradation of FBPase. Consistent with this observation, the Δ*ssa1*Δ*ssa2* strain exhibited a significant decrease in the rate of FBPase degradation (Fig. 4 E). In contrast, the Δ*ssa1*Δ*ssa3* strain degraded FBPase with similar kinetics to WT strains (Fig. 4 F). Thus, this again suggests that Ssa2p is required for FBPase degradation, whereas Ssa1p is not.

To determine whether the Δ*ssa1*Δ*ssa2* FBPase degradation defect could be corrected by the *SSA2* gene, this strain was transformed with a plasmid containing *SSA2* on a GAL-inducible promoter. Cells were grown in low glucose media in the absence (Fig. 5 A) or presence (Fig. 5 B) of the inducing agent (2% galactose). Cells were then shifted to glucose containing media and examined for the rate of FBPase degradation (see Fig. 5 E for a quantitation of these results). The expression of Ssa2p in the Δ*ssa1*Δ*ssa2* double-deletion strain resulted in the restoration of this strain to a WT FBPase degradation phenotype (Fig. 5 B). In contrast, the induction of Ssa1p did not overcome the mutant phenotype (Fig. 5 D). Furthermore, as expected, when the strains were incubated in the absence of the inducing agent, they both exhibited a mutant phenotype (Fig. 5, A and C). Therefore, this offers further evidence that Ssa2p plays an important role in FBPase degradation, while Ssa1p apparently does not.

The role that Ssa2p plays in the import of FBPase into vesicles was further investigated through the use of our in vitro assay. Yeast strains containing single deletions of *SSA1*, *SSA2*, *SSA3*, or *SSA4* were shifted to glucose containing media for 20 min. The cells were harvested and cytosol and Vid vesicles were generated as described above. Neither the individual cytosol nor vesicle fractions were competent for FBPase import from any of the strains (Fig. 6). However, the combination of Δ*ssa1* cytosol and vesicles resulted in a significant amount of import (Fig. 6 A).

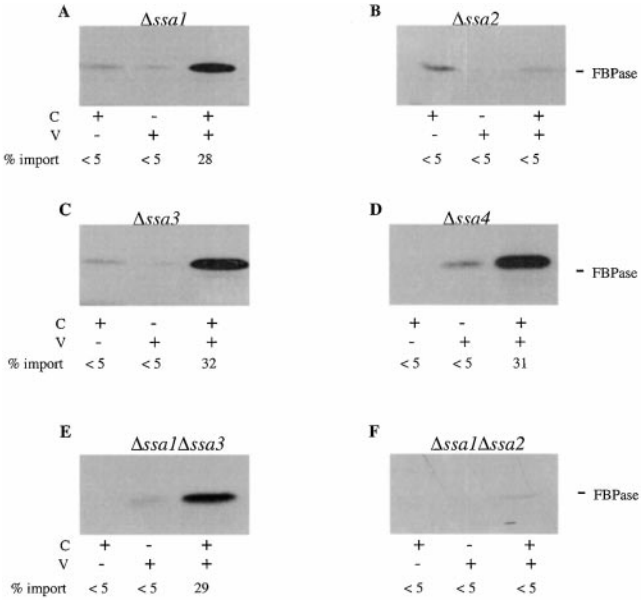

Figure 6. In vitro FBPase import is defective in Δ*ssa2* cells. Yeast Δ*ssa1* (A), Δ*ssa2* (B), Δ*ssa3* (C), and Δ*ssa4* (D) single mutation strains, and Δ*ssa1*Δ*ssa2* (E) and Δ*ssa1*Δ*ssa3* (F) double mutants were grown in YPKG media for 2 d and then shifted to YPD for 20 min. Cytosol (C) and vesicle (V) fractions were generated as described in Materials and Methods and were used in the FBPase import reaction.

Likewise, fractions derived from the Δ*ssa3* (Fig. 6 C) or Δ*ssa4* strains (Fig. 6 D) were competent for FBPase import. Thus, these fractions behave in a similar manner to cytosol and Vid vesicles derived from WT yeast strains. In contrast, the combination of Δ*ssa2* cytosol and vesicles did not support FBPase import (Fig. 6 B), offering further evidence that Ssa2p is required for import of FBPase into vesicles. As expected, cytosol and vesicles from the

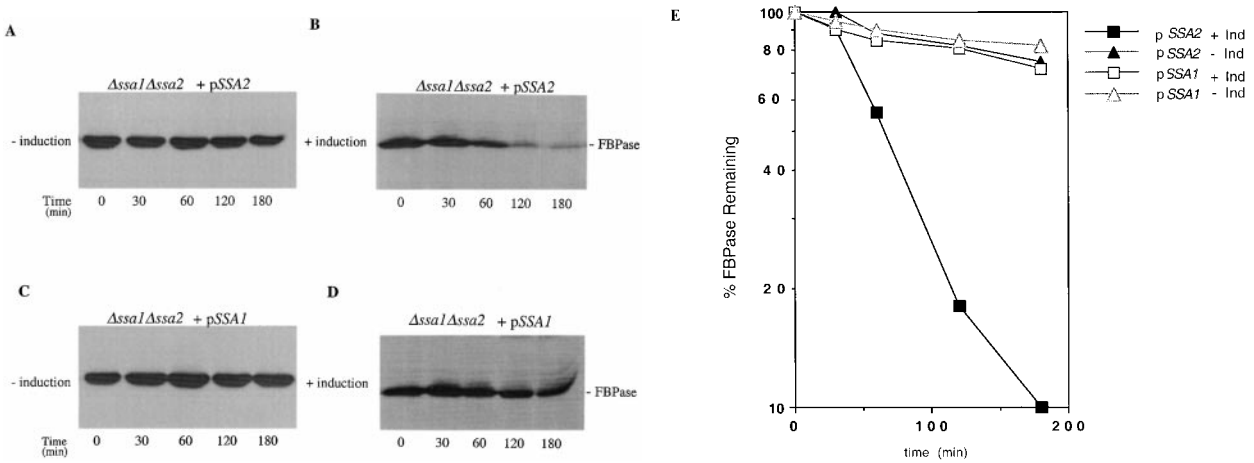

Figure 5. FBPase degradation can be restored by inducing Ssa2p expression in Δ*ssa1*Δ*ssa2* mutants. The Δ*ssa1*Δ*ssa2* strain was transformed with plasmids containing either *SSA2* (A and B) or *SSA1* (C and D) on a GAL inducible promoter. Strains were grown in synthetic media containing 0.5% glucose either in the presence (B and D) or absence (A and C) of the inducing agent (2% galactose). Cells were then shifted to glucose rich SD media for various times. The cells were harvested and examined by SDS-PAGE and Western blot analysis using an anti-FBPase antibody. E, The percentage of FBPase remaining at the various time points was quantitated as described above.

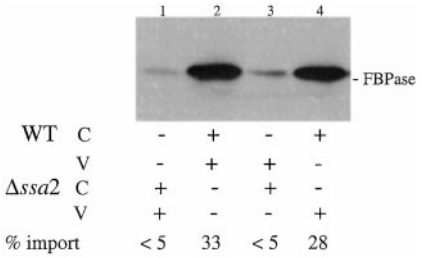

Figure 7. The Δ*ssa2* strain contains cytosol that is defective for FBPase import. Cytosol (C) and Vid vesicles (V) generated from WT and Δ*ssa2* strains was used in FBPase import reactions. Lane 1, import reaction using cytosol and vesicles from the Δ*ssa2* strain. Lane 2, reaction using cytosol and vesicles from the WT strain. Lane 3, vesicles from the WT strain were mixed with cytosol from the Δ*ssa2* strain. Lane 4, vesicles from the Δ*ssa2* strain were combined with cytosol from the WT strain.

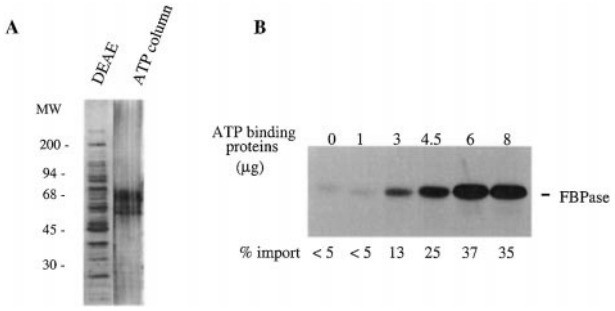

Figure 8. ATP binding proteins stimulate FBPase import in Δ*ssa2* cells. A, ATP binding proteins were purified from WT cells grown in YPD media as described in Materials and Methods. The cells were lysed and the postnuclear supernatant was passed over a DEAE column. DEAE binding proteins were eluted with buffer containing 300 mM KOAc and 1 mM MgATP. The fractions containing protein were pooled and then passed over an ATP agarose column. The ATP binding proteins were eluted with 10 mM MgATP and quantitated via SDS-PAGE and Coomassie blue staining. B, The FBPase import reaction was conducted using cytosol and vesicles generated from Δ*ssa2* cells. Various concentrations of ATP binding proteins were added to the reaction mixture and examined for their ability to induce FBPase import.

Δ*ssa1*Δ*ssa3* strain exhibited WT characteristics (Fig. 6 E), whereas the Δ*ssa1*Δ*ssa2* in vitro components were defective in FBPase import (Fig. 6 F).

Cytosol Lacking Ssa2p Reduces FBPase Import

The defect in FBPase import observed for Δ*ssa2* yeast strain could be due to a number of factors. For example, Ssa2p may play a direct role in vesicular import. Alternatively, Ssa2p might exert its effect via an indirect action on other proteins that are essential for import. Finally, Ssa2p might play a role in the formation or function of the vesicles, perhaps binding to the surface of the vesicles or performing some function in the lumen of the vesicle. Our previous results using cytosol depleted of ATP binding proteins indicated that cytosolic factors play an important role in the process of FBPase import. However, whether or not these cytosolic factors included Ssa2p was not established. Therefore, to better characterize where Ssa2p exerts its effect, we began a series of experiments using various combinations of cytosol and vesicles derived from WT and Δ*ssa2* strains (Fig. 7). As shown previously, the combination of the WT cytosol and WT Vid vesicles resulted in a significant amount of FBPase import (Fig. 7, lane 2), indicating that both the cytosolic and vesicle fractions were competent for FBPase import. In contrast, the combination of Δ*ssa2* cytosol and Δ*ssa2* Vid vesicles did not support import (Fig 7, lane 1), indicating that either one or both of these fractions was incompetent for FBPase import. To distinguish between these possibilities, WT cytosol was mixed with Δ*ssa2* vesicles or WT vesicles were mixed with Δ*ssa2* cytosol. The combination of WT cytosol and Δ*ssa2* Vid vesicles resulted in FBPase import (Fig. 7, lane 4). This indicates that competent vesicles can be formed in the absence of Ssa2p. On the other hand, cytosol from the Δ*ssa2* strain failed to support FBPase import into competent WT vesicles (Fig. 7, lane 3). Thus, it appears that Ssa2p must be present in the cytosol for FBPase import to occur.

Purified Recombinant Ssa2p Stimulates FBPase Import

To further verify the role that Ssa2p plays in the import of FBPase, we began a series of experiments in which purified proteins were added to our in vitro assay. Cytosol from WT cells was passed over a DEAE column as an initial purification step. Selected DEAE fractions enriched for Ssa2p were pooled and applied to an ATP agarose column. After extensive washes, the ATP binding material was eluted with ATP and fractions were examined by SDS-PAGE and silver stain (Fig. 8 A). When the cytosol and vesicle fractions from Δ*ssa2* cells were mixed, there was no detectable import of FBPase. However, if ATP binding proteins were added, we now saw an increase in the amount of FBPase import (Fig. 8 B). This was a dose-dependent effect, with the optimal effect being observed after the addition of 6 μg of protein.

The ATP binding proteins used in the preceding experiment contained Ssa proteins as determined by Western blot analysis using an antibody that recognizes both Ssa1p and Ssa2p (data not shown). Therefore, we suspected that Ssa2p was responsible for the restoration of FBPase import. However, we could not rule out the possibility that other ATP binding proteins might be responsible for this effect. Therefore, in our next experiments, we attempted to purify Ssa2p to utilize this purified protein in our in vitro assay. As a control, Ssa1p was also purified to ascertain the specificity of the effect. The *SSA1* and *SSA2* genes were fused with a HIS6 coding DNA. Yeast were then transformed with these constructs and the resulting tagged proteins were purified using a nickel column. A relatively homogeneous protein profile was obtained using this procedure (Fig. 9 A), although there was some degree of Ssap degradation, as indicated by the faster migrating bands present in both silver stained gels and Western blots (data not shown). When purified, Ssa2p was added to the cytosol and vesicle fractions isolated from the Δ*ssa2* strain, there was a significant increase in the amount of FBPase

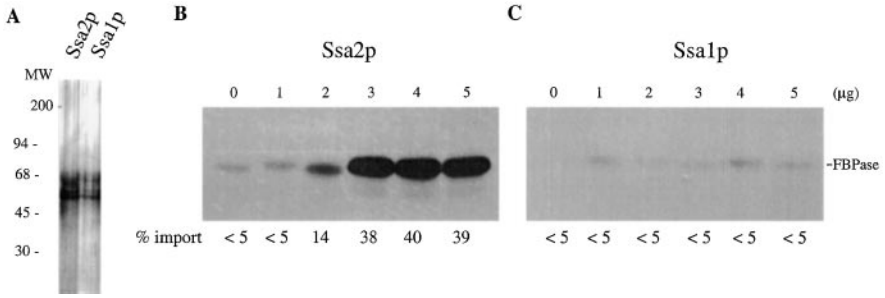

Figure 9. Purified recombinant Ssa2p stimulates FBPase import into Δ*ssa2*-derived Vid vesicles. A, Recombinant Ssa1p and Ssa2p were purified from yeast as described in Materials and Methods. Proteins were visualized via SDS-PAGE and silver staining. B and C, The FBPase import reaction was conducted using cytosol and vesicles generated from Δ*ssa2* cells. B, Purified Ssa2p was added to the reaction mixture at various concentrations to determine the effect on FBPase import. C, Purified Ssa1p was added to the reaction mixture at the same concentrations as a control.

import. This effect was again concentration-dependent, with the optimal response being observed after the addition of 3 μg of purified protein. In contrast, the addition of purified Ssa1p (Fig. 9 C) or BSA (data not shown) had no effect on import, indicating that the results were specific for Ssa2p. Taken together, these results indicate that Ssa2p plays an import role in the import of FBPase into vesicles.

Ssa2p Is Necessary, but Not Sufficient for FBPase Import

In the preceding experiments, we have demonstrated that cytosol is a necessary component for FBPase import into Vid vesicles. We have also identified Ssa2p as an essential cytosolic component for proper import. However, we did not know whether Ssa2p is sufficient to drive import into the vesicles, or whether other cytosolic factors are also required. As an initial control, we reconfirmed that Δ*ssa2* cytosol (Fig. 10, lane 1), vesicles (Fig. 10, lane 2), or the combination of cytosol and vesicles (Fig. 10, lane 3) did not support FBPase import. Next, we tested whether Ssa2p could support import by the individual fractions. As expected, the combination of Ssa2p and cytosol did not result in FBPase import in the absence of Vid vesicles (Fig. 10, lane 4). Likewise, the addition of Ssa2p to vesicles did not support FBPase import in the absence of cytosol (Fig. 10, lane 5). However, the combination of Ssa2p, cytosol, and vesicles did induce the import of FBPase (Fig. 10, lane 6).

Molecular chaperones can protect proteins from proteolytic degradation under certain in vitro circumstances. Therefore, the combination of exogenously added Ssa2p and endogenous cytosolic chaperones might protect FBPase from proteinase K degradation instead of stimulating the import of the protein into vesicles. However, as shown in Fig. 10 A, lane 4, the combination of Ssa2p and cytosol did not protect FBPase from proteolytic degradation. Likewise, Ssa2p did not protect FBPase from degradation in the absence of cytosol or vesicles (Fig 10 A, lane 7). Therefore, we conclude that any protection exhibited in our in vitro assay is most likely due to import into vesicles, and is not a direct protective effect from proteolysis. Additional support for this idea is the observation that the addition of Triton X-100 results in the complete degradation of FBPase, even when cytosol, vesicles, and Ssa2p are included in the reaction mixture (Fig. 10 A, lane 8).

The results from the preceding experiments were further verified using cytosol and vesicles derived from a WT

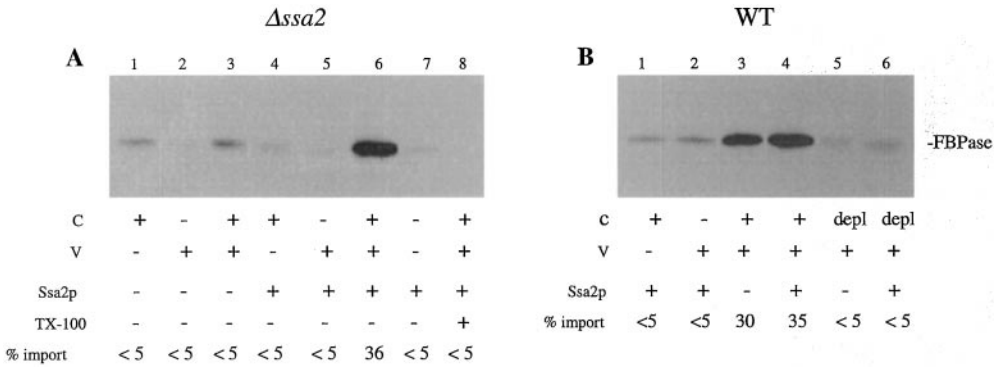

Figure 10. Ssa2p does not drive import of FBPase in the absence of cytosol. A, The FBPase import reaction was conducted using cytosol and vesicles generated from Δ*ssa2* cells. Cytosol (lane 1), vesicles (lane 2), or the combination of cytosol and vesicles (lane 3) did not support FBPase import. The addition of purified Ssa2p to cytosol (lane 4) or vesicles (lane 5) did not support FBPase import, but the addition of Ssa2p to the complete reaction mixture did (lane 6). Purified Ssa2p did not protect FBPase in the absence of added cytosol and vesicles (lane 7). The addition of Triton X-100 resulted in the degradation of FBPase, even in the presence of cytosol, vesicles, and Ssa2p (lane 8). B, The FBPase reaction was conducted using cytosol and vesicles generated from WT cells. Ssa2p did not drive the import of FBPase into cytosol (lane 1) or vesicles (lane 2). The addition of Ssa2p to the complete reaction mixture (lane 4) enhanced the amount of FBPase import slightly above control levels (lane 3). The depletion of ATP binding proteins inhibited the import of FBPase (lane 5). The addition of Ssa2p did not correct this defect (lane 6) indicating that other ATP binding proteins are also necessary for proper FBPase import.

yeast strain (Fig. 10 B). Once again, neither cytosol (Fig. 10 B, lane 1) nor vesicles (Fig. 10 B, lane 2) would support FBPase import when combined with Ssa2p. Thus, it appears that Ssa2p is necessary, but not sufficient for the import of FBPase into Vid vesicles. Accordingly, there must be multiple cytosolic proteins that are required for the import of FBPase into Vid vesicles. To determine whether other cytosolic ATP binding proteins are involved, we repeated our earlier experiments in which ATP binding proteins were depleted from the cytosol. As shown previously, the depletion of ATP binding proteins from the cytosol resulted in the inhibition of FBPase import (Fig. 10 B, lane 5). However, the addition of purified Ssa2p to the reaction mixture did not drive import (Fig. 10 B, lane 6), in contrast to our previous results where the addition of ATP binding proteins did stimulate import (see Fig. 3 B). Thus, it appears that there are additional ATP binding proteins in the cytosol that are required for the import of FBPase into Vid vesicles.

Discussion

Recent evidence suggests that FBPase is targeted to a novel type of vesicle during the degradative process. These Vid vesicles have been purified to near homogeneity and were found to contain FBPase after stimulation with glucose (Huang and Chiang, 1997). Kinetic studies demonstrated that FBPase targeting to Vid vesicles occurs before entry of the protein into the vacuole. Therefore, based upon these and other results, we suspect that there are at least two types of proteins that are involved in FBPase trafficking. The first group may play a role in the sequestration of FBPase into Vid vesicles, whereas the second group is involved in FBPase trafficking from the vesicles to the vacuole. Previously, we identified Vid24p, a protein that appears to validate a part of that prediction (Chiang and Chiang, 1998). Vid24p is induced by glucose, localizes to the vesicles as a peripheral protein and plays an important role in FBPase trafficking from the vesicles to the vacuole. A mutation of the *VID24* gene results in the sequestration of FBPase in Vid vesicles, and prevents the trafficking of FBPase to the vacuole. In the present study, we have demonstrated that the Ssa2p protein is involved in the initial import of FBPase into Vid vesicles. Thus, we have identified at least one protein involved in each of the two predicted trafficking steps for FBPase degradation.

A variety of in vitro systems have been developed for the reconstitution of protein sorting and protein transport into organelles. This includes translocation of proteins into the ER (Brodsky et al., 1993), movement of proteins from the ER to the Golgi body (Beckers et al., 1987; Bednarek et al., 1995) and intra–Golgi body and late Golgi body to vacuole transport (Balch et al., 1984; Vida et al., 1990; Graham and Emr, 1991). Likewise, the import of proteins into various organelles, such as the nucleus (Schlenstedt et al., 1993), mitochondria (Schmitt et al., 1995), and peroxisomes (Zhang and Lazarow, 1996) have been reproduced in vitro. Finally, aspects of the protein degradatory pathway, including components involved in lysosomal and vacuolar import, have been reconstituted in vitro. Along these lines, we have reconstituted the glucose-induced import of FBPase into the yeast vacuole in a semi-intact cellular system (Shieh and Chiang, 1998). In the present study, we developed an in vitro system to study the import of FBPase into Vid vesicles, a process that occurs before the targeting of FBPase into the vacuole. This system utilizes isolated Vid vesicles (200,000-*g* pellet) and cytosol (200,000-*g* supernatant) and can faithfully reconstitute the import of FBPase into a membrane-protected structure. Therefore, this system would appear to be ideal for studies examining one of the important steps involved in FBPase degradation.

Cytosol was required for the uptake of FBPase into the vesicles in our in vitro system. One likely explanation is that cytosolic proteins play a required role in FBPase import. Since FBPase is located in the cytosol before glucose shift, this protein may interact directly with other cytosolic proteins before its trafficking to the vesicles. Along these lines, hsc73 binds to protein substrates that are destined to be degraded in lysosomes (Chiang et al., 1989; Terlecky et al., 1992; Cuervo and Dice, 1996). In addition, hsc73 stimulates in vitro degradation of substrate proteins in isolated lysosomes (Terlecky et al., 1992; Cuervo and Dice, 1996). Therefore, FBPase may be recognized by a constitutive Hsp before its import into the vesicles. In the present study, we have demonstrated a role for the Hsp Ssa2p in the import of FBPase into vesicles. Yeast Δ*ssa2* knockouts exhibit a defect in the degradation of FBPase after a shift from a low glucose to a high glucose circumstance. Furthermore, cytosol isolated from this strain was not functional for in vitro import of FBPase. Interestingly, Ssa2p is required as a soluble cytosolic component for import, but it is not required for the formation of competent Vid vesicles. Vid vesicles purified from Δ*ssa2* cells are competent for import if they are incubated with WT cytosol or with cytosol that contains the Ssa2p protein. In contrast, WT vesicles cannot import FBPase if they are incubated with cytosol that lacks the Ssa2p protein.

Although Ssa2p is a required cytosolic component, it is not the only cytosolic protein necessary for FBPase import into Vid vesicles. For example, the combination of purified recombinant Ssa2p and import competent Vid vesicles did not import FBPase unless cytosol was included in the reaction. Accordingly, we surmise that a number of other cytosolic proteins may be essential for the FBPase import process. The identification of these proteins was beyond the scope of the present study. However, we suspect that a number of ATP binding proteins may be involved. As shown in Fig. 3 B, the depletion of ATP binding proteins from the cytosolic fraction resulted in the inhibition of FBPase import. Although Ssa2p was identified as one of the proteins depleted in this process, the readdition of purified recombinant Ssa2p did not restore import competence to this cytosol. Thus, it appears that other ATP binding proteins are also required for FBPase import.

Somewhat surprisingly, Ssa1p did not appear to play a role in FBPase import. As noted previously, *SSA1* and *SSA2* are ~97% homologous and are known to share many overlapping functions. However, we have multiple lines of evidence that suggest that they do not play similar roles in the degradation of FBPase. First, the deletion of the *SSA1* gene did not have any apparent effect on the kinetics of FBPase degradation after a glucose shift. This

was observed for both the Δ*ssa1* and Δ*ssa1*Δ*ssa3* strains. In contrast, both the Δ*ssa2* and Δ*ssa1*Δ*ssa2* strains exhibited defective FBPase degradation kinetics. Again, this defect was attributed to the lack of Ssa2p, since the induced expression of Ssa2p in the Δ*ssa1*Δ*ssa2* strain corrected the degradation defect, whereas the induced expression of Ssa1p did not.

The role that Ssa2p played in FBPase import was further confirmed with our in vitro assay. Cytosol and vesicles derived from the Δ*ssa2* strain would not support FBPase import, whereas cytosol and vesicles derived from the Δ*ssa1*, Δ*ssa3*, or Δ*ssa4* strains did import FBPase. Furthermore, the addition of purified recombinant Ssa2p to the Δ*ssa2* reaction mixture restored FBPase import, whereas the addition of purified recombinant Ssa1p did not. Therefore, we conclude that Ssa2p is required for FBPase import, both in vivo and in vitro.

The screening of genetic mutations is a powerful tool to identify genes that are involved in various cellular processes. However, the process is not necessarily all-inclusive, as demonstrated by the present study. Members of the Ssa chaperone family were not identified in our initial genetic screen for FBPase degradation mutants, although Ssa2p clearly plays a role in the FBPase degradation process. Therefore, a combination of genetic and biochemical methodologies will no doubt be necessary to identify most of the components involved in the trafficking and degradation of the FBPase protein. Further studies using the purified vesicles as an in vitro assay system should be instrumental in elucidating the mechanisms involved in the FBPase degradation pathway.

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