Biochemical Analysis of Fructose-1,6-bisphosphatase Import into Vacuole Import and Degradation Vesicles Reveals a Role for UBC1 in Vesicle Biogenesis*

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When Saccharomyces cerevisiae are shifted from medium containing poor carbon sources to medium containing fresh glucose, the key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) is imported into Vid (vacuole import and degradation) vesicles and then to the vacuole for degradation. Here, we show that FBPase import is independent of vacuole functions and proteasome degradation. However, FBPase import required the ubiquitin-conjugating enzyme Ubc1p. A strain containing a deletion of the UBC1 gene exhibited defective FBPase import. Furthermore, FBPase import was inhibited when cells overexpressed the K48R/K63R ubiquitin mutant that fails to form mult ubiquitin chains. The defects in FBPase import seen for the ∆ubc1 and the K48R/K63R mutants were attributed to the Vid vesicle fraction. In the ∆ubc1 mutant, the level of the Vid vesicle-specific marker Vid24p was reduced in the vesicle fraction, suggesting that UBC1 is required for either Vid vesicle production or Vid24p binding to Vid vesicles. However, the K48R/K63R mutant did not prevent Vid24p binding to Vid vesicles, indicating that ubiquitin chain formation is dispensable for Vid24p binding to these structures. Our results support the findings that ubiquitin conjugation and ubiquitin chain formation play important roles in a number of cellular processes including organelle biogenesis.

The vacuole of the yeast Saccharomyces cerevisiae is homologous to the lysosome of higher eucaryotes and as such, plays an important role in protein degradation (1–4). The function of the vacuole requires the targeting of a number of vacuole resident proteins into this organelle. These proteins are sorted to this organelle by several mechanisms and require the assistance of numerous genes. For example, targeting of the vacuole luminal protein carboxypeptidase Y (CPY) from the late Golgi requires more than 40 VPS genes (1–4).

Proteins and organelles can be delivered to the vacuole from the cytoplasm by the microautophagy or macroautophagy pathways (5–11). Regulation of the autophagic process can have important consequences on cellular physiology. For example, the tumor suppressor gene beclin-1 is homologous to APG6/ VPS30 and induces autophagy in yeast and mammalian cells. Therefore, a decrease in autophagic protein degradation may contribute to the development or progression of human malignancy (13).

A nonselective macroautophagy pathway is induced when S. cerevisiae are starved of nitrogen (5–11). This pathway requires a novel ubiquitin-like conjugating system (14). Furthermore, this pathway also overlaps with the cytoplasm to vacuole targeting pathway for targeting aminopeptidase I from the cytoplasm (5–11). Aminopeptidase I trafficking to the vacuole occurs by two routes (11). Under normal growth conditions, aminopeptidase I is targeted to the vacuole by cytoplasm to vacuole targeting vesicles. When cells are starved of nitrogen, however, aminopeptidase I is delivered to the vacuole by the macroautophagy pathway (11). Recent evidence suggests that the cytoplasm to vacuole targeting pathway also shares components with the peroxisome microautophagy pathway (15–17).

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 (18). When fresh glucose is added to the medium, however, FBPase is targeted to the vacuole and degraded (19, 20). This redistribution of FBPase to the vacuole has been observed by immunofluorescence microscopy, cell fractionation, and electron microscopy (19, 20). More recently, FBPase targeting to the vacuole has been reconstituted in vitro using permeabilized yeast cells incubated with purified radiolabeled FBPase in the presence of ATP, an ATP regenerating system and cytosolic proteins (21).

FBPase is imported into a novel type of Vid (vacuole import and degradation) vesicle prior to its uptake by the vacuole (22). These vesicles have been purified to near homogeneity from wild-type cells (22). The identification of Vid vesicles in the FBPase degradation pathway suggests that this pathway can be divided into at least two steps. The first step is the targeting and sequestration of FBPase into Vid vesicles. The second step is the delivery of FBPase from Vid vesicles to the vacuole for degradation.

Since Vid vesicles do not contain markers from known organelles, they may represent a novel transport structure, although it is possible that Vid vesicles are derived from existing structures. Thus far, the heat shock protein Ssa2p is the only molecule that has been shown to play a role in the import of FBPase into Vid vesicles (23). To identify more molecules involved in this process, we analyzed the import of FBPase into Vid vesicles using various inhibitors and mutants. We found...
that FBPase import was not affected by inhibitors or mutants that block vacuole acidification, vacuole proteolysis, or proteasome degradation. However, FBPase import did require ubiquitin chain formation and the ubiquitin conjugation enzyme Ubc1p. The ∆ubc1 mutant contained defective vesicles, but competent cytosol. Furthermore, FBPase import was inhibited when cells overexpressed a ubiquitin mutant (K48R/K63R) that prevents the formation of multubiquitin chains. The defect of the K48R/K63R mutant was associated with Vid vesicles, indicating that ubiquitin chain formation is required to produce competent Vid vesicles.

In the absence of the UBC1 gene, the level of the Vid vesicle-specific marker Vid24p was reduced in the Vid vesicle pellet fraction, suggesting that UBC1 is required for Vid vesicle production. Alternatively, Vid24p binding to Vid vesicles may be compromised in the absence of ubiquitination. However, overproduction of the K48R/K63R mutant did not prevent Vid24p binding to Vid vesicles. Since ubiquitin chain formation is necessary for Vid vesicle function, but is dispensable for Vid24p binding to Vid vesicles, these results are consistent with the hypothesis that Vid vesicle function is regulated by ubiquitin conjugation and ubiquitin chain formation. Thus, our work complements previous studies in which ubiquitin conjugation is important for peroxisome biogenesis (24), mitochondrial inheritance (25), mitochondrial targeting (26), and receptor-mediated endocytosis (27–31).

EXPERIMENTAL PROCEDURES

Yeast Strains, Chemicals, and Antibodies—S. cerevisiae strains used in this study are listed in Table I. For the in vitro experiments, the endogenous FBP1 gene was deleted and a known quantity of purified FBPase was added to the reaction. To produce the FBP1 null strain, the FBP1 gene was cloned into pBR322 to yield the plasmid pJS31. The FBP1 deletion construct was generated by deleting 90% of the FBP1 gene from pJS31 with StuI and religating with a Linear containing fragment which was produced by digestion of the YEP13 plasmid with BglII. The deletion construct was then digested with BamHI and HindIII and transformed into yeast strains using the standard lithium acetate method. The deletion of FBP1 was confirmed by Western blotting with anti-FBPase antibodies.

A pep4 null mutation was produced using the pTS15 plasmid provided by Dr. T. Stevens (University of Oregon). This plasmid was digested with EcoRI and XhoI to disrupt the FEP4 locus (32). The defect in the pep4 null strains was confirmed by the accumulation of the p2 form of CPY intracellularly. A strain with a null mutation of the VID1 gene was also utilized. The VID1 gene is identical to the ISE1 or ERG6 gene. The gene was amplified by polymerase chain reaction and cloned into a TA cloning vector (Invitrogen) using a 5′ primer AGCGGC-GGCCGATGGGGATGAAAGACAAATGGGAGAA and a 3′ primer GACGCGCGCCGCTAGTTGCTTCTTGGGAAGTTTGGG. A deletion construct was produced by removing 80% of the gene via KpnI and PmeI digestion, and religating with a UR3 containing fragment produced by digesting the YIP352 plasmid with SmaI and HpaI. The resultant construct was linearized with NotI and transformed into a wild type strain. The deletion was confirmed by polymerase chain reaction analysis. The pUB223 plasmid containing the Myc-tagged K48R/K63R ubiquitin mutant and the Ub-Pro-βgal plasmid (33, 34) were obtained from Dr. D. Finley (Harvard Medical School).

YPD is a complete medium (10 g/liter of Bacto-yeast extract, 20 g/liter of Bacto-peptone, Difco Labs Inc.) supplemented with 20 g/liter dextrose (Fisher Scientific). YPKG contained 10 g/liter Bacto-yeast extract, 20 g/liter Bacto-peptone, 10 g/liter potassium acetate, and 5 g/liter dextrose. Synthetic minimal medium consisted of 0.67 g/liter nitrogen base without amino acids, supplemented with 5 g/liter casamino acids, 40 mg/liter adenine, 60 mg/liter leucine, and 20 g/liter dextrose. Inhibitors used in this study included ATP-S, N-ethylmaleimide, brefeldin A, bafilomycin A, and concanamycin A and were purchased from Sigma. MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal) and ethylmaleimide were gifts from Dr. A. Goldberg (Harvard Medical School). Tran35S-labeled (10 Ci/mmol) was obtained from ICN. Rabbit anti-FBPase and rabbit anti-Cpy polyclonal antibodies were raised by Berkeley Antibody Co. (Berkeley, CA) using purified FBPase and CPY (Sigma). Mouse and rabbit anti-Myc antibodies were purchased from Berkeley Antibody Co. Mouse anti-β-galactosidase antibodies were purchased from Promega.

The FBPase Import Assay—The FBPase import assay was performed according to Shiieh and Chiang (21). In a typical experiment, the reaction mixture (100 µl) contained 3 A540 units of semi-intact cells, 11 µg of 35S-FBPase, an ATP regenerating system (0.5 mM ATP, 0.2 mM MgCl2, 10 µCi phosphoprotein, 40 mM creatine phosphate), and 0.5 mM MgCl2. The mixture was incubated at 30 °C for the indicated times, after which 0.8 mg/ml proteinase K was added to identify the
intact cells and cytosol were prepared as described (21). FBPase import was shifted to glucose for 0, 60, and 120 min. Total lysates from these cells were solubilized in SDS buffer, separated by SDS-PAGE and FBPase degradation was followed in these cells. B, both Δise1 (HLY208) and Δvid24 (HLY232) mutants were shifted to glucose for 20 min. Semi-intact cells and cytosol were prepared as described (21). FBPase import was measured for 0, 10, 20, and 30 min in the absence or presence of ATP and cytosol. The % FBPase import is indicated.

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FBPase Import in Vitro—To biochemically analyze FBPase import into Vid vesicles, we used an in vitro system that reproduces the defects seen for mutants affecting the FBPase degradation pathway. For example, both the Δvid1 (Δise1) and Δvid24 mutants inhibit the degradation of FBPase in vitro (Fig. 1A). However, these mutations affect different steps in the FBPase degradation pathway. The Δvid24 mutant strain imports FBPase into Vid vesicles normally, but this mutation blocks the trafficking of Vid vesicles to the vacuole. As such, this mutation results in the accumulation of FBPase in Vid vesicles (37). On the other hand, a mutation of the VID1 gene (a gene that is identical to the ISE1 or ERG6 gene) blocks FBPase import into Vid vesicles (38) and serves as a negative control for in vitro import.

To examine FBPase import in the Δise1 and Δvid24 strains, the endogenous FBP1 gene was deleted so that a known quantity of radiolabeled, purified FBPase could be added and followed in the in vitro system. Each strain was glucose starved and then shifted to glucose containing medium prior to their conversion to semi-intact cells. Purified FBPase was incubated with semi-intact cells in the absence or presence of ATP, an ATP regenerating system and cytosol. At selected times, protease K was added to digest the FBPase that was not protected in a membrane-sealed compartment. In the absence of both ATP and cytosol, FBPase import into the Δvid24 semi-intact cells was minimal (Fig. 1B). In the presence of ATP and cytosol, however, FBPase import increased in a time-dependent manner. When quantitated, ~25–35% of the total added FBPase was protease K protected after 30 min of import. In contrast, the Δise1 mutant had background levels of FBPase import either in the presence or in the absence of ATP and cytosol (Fig. 1B).

The Δise1 Mutant Contains Defective Vesicles—The defect of FBPase import seen for the Δise1 mutant could result from an inability of cytosol to stimulate FBPase import or an inability of Vid vesicles to take up FBPase. To determine the site of this defect, we performed an in vitro assay using various combinations of semi-intact cells and cytosol from the Δise1 and Δvid24 mutants. When the Δise1 semi-intact cells were used, FBPase import was defective regardless of whether the cytosol was isolated from the Δise1 (Fig. 2, lane 1) or the Δvid24 mutants (lane 3). By contrast, FBPase import into the Δvid24 semi-intact cells was observed when cytosol was prepared from either the Δvid24 mutant (lane 2) or the Δise1 mutant (lane 4). This experiment suggests that the Δise1 mutant strain has competent cytosol that can stimulate FBPase import into competent Vid vesicles. However, the Δise1 mutant contains defective vesicles that cannot support FBPase import, even when combined with import-competent cytosol.

FBPase Import Is Independent of Vacuole Proteolysis and Vacuole Acidification—Next, we utilized our in vitro assay to investigate whether FBPase import into Vid vesicles was dependent on other cellular processes such as vacuole proteolysis or vacuole acidification. The PEP4 gene is required for the maturation of several major vacuolar proteinases including CPY. Hence, the deletion of the PEP4 gene renders cells defective in vacuolar proteolysis (1, 3). In wild type cells, CPY is synthesized as prepro-CPY and then translocated into the endoplasmic reticulum where it is glycosylated to p1-CPY in the endoplasmic reticulum (1–4). CPY is further modified in the Golgi to p2-CPY and finally processed to the mature form in the vacuole (1–4). Therefore, the deletion of the PEP4 gene resulted in the accumulation of p2-CPY in the Δise1Δpep4 and Δvid24Δpep4 strains (Fig. 3A). When FBPase import was measured, the level was low in the Δise1 single mutant (Fig. 3B, lane 1) and there was no significant increase in the FBPase import in the Δise1Δpep4 double mutant (lane 2). Likewise, there was no significant change in FBPase import in the
pulse-chase experiments in the intact cells were prepared and FBPase import was measured in the (HLY217) were shifted to glucose for 20 min. The cytosol and semi-pep4Dvid24strains. DPEP4 in FBPase import. The trafficking to the vacuole. A model that FBPase import into Vid vesicles occurs prior to ubiquitination of misfolded or unassembled proteins in the endoplasmic reticulum degradation pathway (45, 46). Therefore, Δubc6 and Δubc7 strains did not inhibit the degradation of short-lived proteins (Fig. 4A). When the Δubc1, Δubc6, and Δubc7 strains were tested for FBPase import, a reduced level of import was observed for Δubc1 (Fig. 4B, lane 1), but not for the Δubc6 and Δubc7 strains (lanes 2 and 3), suggesting a specific role for UBC1 in the import process. When the Δvid24Dpep4 double mutant (lane 4) as compared with the Δvid24 single mutant (lane 3). Since uptake of FBPase by Vid vesicles is independent of the PEP4 gene, this supports our model that FBPase import into Vid vesicles occurs prior to trafficking to the vacuole.

As is shown in Fig. 1, the addition of ATP and cytosol stimulates FBPase import into Vid vesicles. This suggests that ATPases and/or ATP hydrolysis (see below) may play some role in FBPase import. The VMA3 gene, which encodes the 16-kDa proteolipid subunit of the membrane sector of the V-ATPase (1, 39), has previously been shown to play a role in autophagy (40). However, when FBPase import was measured in the vma3 deletion mutant, there was no significant defect (Fig. 3B, lane 5). Therefore, V-ATPase is not essential for FBPase import into Vid vesicles.

FBPase Import Requires the UBC1 Gene—Ubiquitination plays an important role in distinct biological functions including DNA repair, protein degradation, organelle biogenesis, and protein trafficking (41, 42). For example, the ubiquitin protein ligase Rsp5p is essential for mitochondrial inheritance and mitochondrial import (25, 26). Rsp5p is also involved in receptor-mediated internalization of Ste2p, Ste3p, and other cell surface proteins (31). In addition, the ubiquitin-conjugating enzyme Ubc10p plays a critical role in peroxisomal biogenesis (24). Ubc10p is one of 13 ubiquitin-conjugating enzymes found in yeast (41, 42). UBC1 are functionally dispensable in the Ubc6 and Ubc7 mutants (43, 44). As expected, the Δubc1 strain displayed a reduced rate of degradation of short-lived proteins as compared with the wild type control (Fig. 4A). In contrast, UBC6 and UBC7 are involved in the ubiquitination of misfolded or unassembled proteins in the endoplasmic reticulum degradation pathway (45, 46). Therefore, Δubc6 and Δubc7 strains did not inhibit the degradation of short-lived proteins (Fig. 4A). When the Δubc1, Δubc6, and Δubc7 strains were tested for FBPase import, a reduced level of import was observed for Δubc1 (Fig. 4B, lane 1), but not for the Δubc6 and Δubc7 strains.

One of the major functions of ubiquitin conjugation is to target proteins for degradation by the proteasome (41, 42). However, ubiquitin conjugation can also have other important functions unrelated to protein degradation (24–31, 41, 42). We investigated whether the proteasome plays a role in FBPase import using the pre1-1pre2-1 proteasome mutant. PRE1 and PRE2 encode subunits of the 20 S core particle of the proteasome and an interaction between Pre1p and Pre2p is necessary for formation of the chymotrypsin-like active site in the proteasome (47, 48). A decrease in the degradation rate of short-lived proteins was observed for the pre1-1pre2-1 mutant strain (Fig. 4A). However, the import of FBPase in the pre1-1pre2-1 mutant was not altered (Fig. 4B, lane 4). Thus, the proteasome is unlikely to be involved in the import process.

Inhibitor Studies—We next investigated whether FBPase import was dependent upon vacuole acidification or proteasome degradation using inhibitors that block these processes (Fig. 5). For these experiments, Δvid24 semi-intact cells and cytosol were preincubated with various concentrations of inhibitors. These concentrations were chosen based upon previous studies demonstrating maximal inhibition in the yeast system (35, 36, 49–51). FBPase, ATP, and an ATP regenerating system were then added to the reaction mixture to commence the import process. The in vitro import of FBPase was inhibited by nonhydrolyzable ATP analogs (Fig. 5A, lane 3). However, N-ethylmaleimide, which inhibits V-ATPase (1) did not affect FBPase import in vitro (lane 4). Likewise, brefeldin A had no effect on in vitro FBPase import (lane 5), even though this inhibitor caused accumulation of p1-CPY in the ies1 (brefeldin A permeable) strain (Fig. 5B, lane 2). FBPase import was also unaffected by the proteasome inhibitors MG132 or β-lactone (Fig. 5A, lanes 6 and 7), although these inhibitors did reduce the degradation of short-lived proteins in vivo (Fig. 5C). Inhibitors that perturb vacuole acidification such as bafilomycin A and concanamycin A (1, 50, 51) also had no effect on FBPase import (Fig. 5A, lanes 8 and 9), but they did reduce the degradation of long-lived proteins in vivo (Fig. 5D). Taken together, the mutant analyses and the inhibitor studies suggest that FBPase import into Vid vesicles is independent of vacuole proteolysis, vacuole acidification, and proteasome degradation. However,
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Fig. 5. The effects of inhibitors on FBPase import. A, FBPase import into semi-intact Δvid24 cells (HLY232) was carried out in the absence (lane 1) or presence (lane 2) of ATP and cytosol or with preincubation of various inhibitors (lanes 3–12). ATP-S (50 μM), N-ethylmaleimide (10 mM), brefeldin A (75 μg/ml), MG132 (100 μM), β-lactone (50 μM), bafilomycin A (20 μM), and concanamycin A (0.3 μM) were added to semi-intact cells and cytosol for 20 min before the addition of FBPase, ATP, and an ATP regenerating system. FBPase import was measured as described. The percentage of FBPase import in semi-intact cells treated with various inhibitors is indicated. B, the addition of brefeldin A caused p1-CPY to accumulate in the ise1 strain (lane 2). C, the degradation of short-lived proteins was inhibited by MG132 and β-lactone. D, inhibitors that perturb the acidification of the vacuole reduced the degradation of long-lived proteins.

If UBC1 is required for Vid vesicle formation, the number of Vid vesicles should be reduced in the Δubc1 mutant. This would be reflected as a decreased level of Vid24p within fractions that contain Vid vesicles. Conversely, if UBC1 is required for the function of the import machinery, the level of Vid24p would not be altered in the Vid vesicle containing fractions. To test these possibilities, the wild type, Δubc1, and Δubc6 strains were shifted to glucose for 180 min. In contrast, FBPase degradation was significantly retarded in the Δubc1 mutant, but was normal in the Δubc6 mutant. Therefore, UBC1 is required for FBPase degradation, whereas UBC6 is not.

We next examined whether the defect in FBPase import observed for the Δubc1 mutant resulted from an inability of cytosol to support FBPase import or an inability of Vid vesicles to take up FBPase. As is shown in Fig. 6A, wild type cells degraded FBPase after a shift to glucose for 180 min. In contrast, FBPase degradation was significantly retarded in the Δubc1 mutant, but was normal in the Δubc6 mutant. Therefore, UBC1 is required for FBPase import, whereas UBC6 is not.

This import does require ATP hydrolysis and the UBC1 gene.

UBC1 Is Necessary for FBPase Import—Since the Δubc1 strain displayed defective FBPase import in vitro, we next determined whether this strain was also defective in FBPase degradation in vivo. As is shown in Fig. 6A, wild type cells degraded FBPase after a shift to glucose for 180 min. In contrast, FBPase degradation was significantly retarded in the Δubc1 mutant, but was normal in the Δubc6 mutant. Therefore, UBC1 is required for FBPase degradation, whereas UBC6 is not.

We next examined whether the defect in FBPase import observed for the Δubc1 mutant resulted from an inability of cytosol to support FBPase import or an inability of Vid vesicles to take up FBPase. As is shown in Fig. 6B, when cytosol and semi-intact cells from the Δubc1 strain were used, FBPase import was impaired (lane 1). By contrast, when cytosol and semi-intact cells from the Δvid24 strain were combined, a high level of FBPase import was observed (lane 2). FBPase import decreased when Δubc1 semi-intact cells were incubated with cytosol from the Δvid24 strain (lane 3). Since the Δvid24 strain contained import competent cytosol, this result indicates that the Δubc1 mutant had defective vesicles. In contrast, the Δubc1 strain appears to contain competent cytosol, because cytosol from the Δubc1 strain supported FBPase import into import competent Vid vesicles in Δvid24 semi-intact cells (lane 4).

The impaired ability of the Δubc1 semi-intact cells to import FBPase could be due to a decrease in Vid vesicle production. Alternatively, the reduced import could result from a defect in the import machinery. In initial experiments, we examined the levels of the Vid vesicle specific marker, Vid24p. Vid24p is induced in response to glucose and a significant portion of this protein is associated with Vid vesicles as a peripheral protein (37). When cells were maintained in low glucose medium (2°C for 0 min), Vid24p was undetectable in total lysates. However, this protein was induced to a similar level after wild type, Δubc1, and Δubc6 strains were shifted to glucose for 20 min (Fig. 7A). Therefore, Vid24p production is not altered in the Δubc1 mutant.
prepared from the wild type strain overexpressing wild type ubiquitin (Fig. 6B, lane 1). By contrast, in vitro FBPane import was significantly reduced when both cytosol and semi-intact cells were prepared from the strain that overproduced the K48R/K63R mutant (lane 2). When cytosol from the K48R/K63R strain was incubated with semi-intact cells from the strain overexpressing wild type ubiquitin, a high level of FBPane import was detected as multiple bands in cells transformed with the Ub-Pro-b-galactosidase plasmid (Fig. 9A, lanes 1–4). As shown by immunoblotting and immunoprecipitation experiments, Ub-Pro-b-galactosidase was present as multiple bands in cells transformed with the Ub-Pro-b-galactosidase plasmid (lanes 5, 6, 9, and 10). However, these bands were not observed in control cells that did not contain the Ub-Pro-b-galactosidase plasmid (lanes 7, 8, 11, and 12). In cells transformed with both Ub-Pro-b-galactosidase and Myc ubiquitin plasmids, multiple Ub-Pro-b-galactosidase bands were detected by anti-Myc antibodies, suggesting that these bands were polyubiquitinated forms of Ub-Pro-b-galactosidase (lane 14). By contrast, no Myc signal could be found in cells that did not harbor the Myc ubiquitin plasmid (lane 13) or in cells

The K48R/K63R Mutant Does Not Prevent Vid24p Binding to Vid Vesicles—As mentioned above, the decreased level of Vid24p in the Δubc1 high speed pellet may result from a reduced number of Vid vesicles, or it may be due to a decreased binding of this protein to Vid vesicles. Accordingly, if ubiquitin chain formation is necessary for Vid24p binding to Vid vesicles,
that did not contain the Ub-Pro-β-galactosidase plasmid (lanes 15 and 16).

To determine whether Vid24p was ubiquitinated, this protein was immunoprecipitated from total lysates of wild type cells and then immunoblotted with anti-Myc antibodies. Vid24p was expressed in wild type cells, but was absent in the Δvid24 strain, as indicated by immunoblotting (Fig. 9B, lanes 5–8) and immunoprecipitation experiments (lanes 9–12). When the precipitated Vid24p was immunoblotted with anti-Myc antibodies, there was no detectable Myc signal (lane 14). Likewise, no ubiquitination of Vid24p could be found in cells that did not contain the Myc ubiquitin plasmid (lane 13), or in the Δvid24 strain (lanes 15 and 16). Furthermore, no ubiquitination of Vid24p could be detected using pulse-chase experiments followed by immunoprecipitation with anti-Vid24p antibodies (data not shown). Therefore, Vid24p is unlikely to be ubiquitinated. This supports our contention that ubiquitination is not required for the function of Vid24p.

Although the site of FBPAse degradation has been a matter of debate (52, 53), a PEP4-dependent degradation of FBPAse was confirmed by an independent research group (54). To examine whether FBPAse was polyubiquitinated, wild type and Δfbp1 strains were transformed with or without the Myc-tagged ubiquitin plasmid using the protocol described by Schork et al. (52). These cells were then shifted to glucose for the indicated times and FBPAse degradation was examined. B, wild type cells overexpressing either wild type ubiquitin (HLY820) or the K48R/K63R ubiquitin mutant (HLY819) were shifted to glucose for 20 min. Cytosol and semi-intact cells from these strains were combined as indicated and in vitro import of FBPAse was performed as described under “Experimental Procedures.” C, total lysates from wild type cells over-expressing either wild type ubiquitin (HLY824) or the K48R/K63R ubiquitin mutant (HLY823) were fractionated by differential centrifugation. The distribution of Vid24p in total (T), high speed pellet (P), and high speed supernatant (S) fractions was examined by Western blotting with anti-Vid24p antibodies.

**DISCUSSION**

In this study, we analyzed FBPAse import into Vid vesicles to identify molecules involved in early stages of the FBPAse degradation pathway. Our results suggest that vacuole proteolysis, vacuole acidification, and proteasome degradation are unlikely to be involved in FBPAse import. The Δis1Δisep4 or Δvid24Δisep4 double mutants did not alter FBPAse import as compared with the Δis1 or Δvid24 single mutants, suggesting that FBPAse import is independent of the PEP4 gene. Furthermore, the vma3 deletion mutant and compounds such as bafilomycin A or concanamycin A that block acidification of the vacuole did not inhibit FBPAse import. Therefore, FBPAse import is independent of the two major vacuole functions, vacuole proteolysis and vacuole acidification. This further supports our model that FBPAse import into Vid vesicles occurs prior to the trafficking to the vacuole.

Our results indicate that the cytosolic ubiquitin-conjugating enzyme Ubc1p is an important regulator of the FBPAse import process. FBPAse import into Vid vesicles is defective in the Δubc1 mutant, but not in the Δubc6 or Δubc7 mutants, suggesting a specific role for UBC1 in the import process. However, this requirement is not linked to proteasome degradation. The pre1-1 pre2-1 proteasome mutant showed normal FBPAse import and proteasome inhibitors such as MG132 and β-lactone had no effect on FBPAse degradation.
Our data show that UBC1 is required for the proper function of Vid vesicles. The Δubc1 mutant contained defective vesicles, but normal cytosol. In the absence of the UBC1 gene, cells may decrease the production of Vid vesicles or reduce the efficiency of the import machinery. In the control wild type and Δubc6 strains, most of the Vid vesicle marker Vid24p was found in fractions containing Vid vesicles. When quantitated, ~90% of the Vid24p was recovered in the Vid vesicle containing pellet fraction in wild type cells. However, in the Δubc1 strain, about 25% of the Vid24p was in the pellet fraction, while most of the Vid24p was in the soluble fraction. The reduced levels of Vid24p in the pellet fraction could result from decreased Vid vesicle production or a decreased binding of Vid24p to Vid vesicles. However, the K48R/K63R mutant did not prevent Vid24p binding to Vid vesicles, even though it inhibited vesicle import. Therefore, polyubiquitination is necessary for FBPase import into Vid vesicles, but does not play an important role in Vid24p binding to Vid vesicles.

Based upon results from this study and from previous studies (23, 37), we have proposed a model for the FBPase degradation pathway (Fig. 10). In the initial step, FBPase is imported into Vid vesicles through a process that requires the presence of the heat shock protein Ssa2p. Following FBPase sequestration inside these structures, the loaded vesicles then traffic to the vacuole via a process controlled by Vid24p. At
present, the site of origin for Vid vesicles is unknown, although the formation of these organelles appears to be regulated by the cytosolic ubiquitin-conjugating enzyme Ubc1p. In the absence of this enzyme, levels of Vid vesicles are reduced and FBPase degradation is compromised. Therefore, we propose that ubiquitination plays an important role in the degradation of FBPase through its effect on the machinery (Vid vesicles) that transports FBPase to the vacuole. Identification of the factors that are polyubiquitinated by Ubc1p may ultimately help identify their sites of action as well as to elucidate how FBPase is imported into Vid vesicles.

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