Degradation of the Gluconeogenic Enzymes Fructose-1,6-bisphosphatase and Malate Dehydrogenase Is Mediated by Distinct Proteolytic Pathways and Signaling Events*

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The key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) is subjected to catabolite inactivation and degradation when glucose-starved cells are replenished with fresh glucose. In various studies, the proteasome and the vacuole have each been reported to be the major site of FBPase degradation. Because different growth conditions were used in these studies, we examined whether variations in growth conditions could alter the site of FBPase degradation. Here, we demonstrated that FBPase was degraded outside the vacuole (most likely in the proteasome), when glucose was added to cells that were grown in low glucose media for a short period of time. By contrast, cells that were grown in the same low glucose media for longer periods of time degraded FBPase in the vacuole in response to glucose. Another gluconeogenic enzyme malate dehydrogenase (MDH2) showed the same degradation characteristics as FBPase in that the short term starvation of cells led to a non-vacuolar degradation, whereas long term starvation resulted in the vacuolar degradation of this protein. The N-terminal proline is required for the degradation of FBPase and MDH2 for both the vacuolar and non-vacuolar proteolytic pathways. The cAMP signaling pathway and the phosphorylation of glucose were needed for the vacuolar-dependent degradation of FBPase and MDH2. By contrast, the cAMP-dependent signaling pathway was not involved in the non-vacuolar degradation of these proteins, although the phosphorylation of glucose was required.

Gluconeogenesis is a highly regulated cellular process by which yeast can produce glucose from alternative nutrient sources such as acetate, amino acids, lactate, and glycerol. When exogenous glucose is available, however, many gluconeogenic enzymes are transcriptionally repressed (1–4) and the existing enzymes are subjected to rapid inactivation and proteolytic degradation (5–9). Extensive studies have been conducted on the catabolite repression of gluconeogenic enzymes at the transcriptional level (10, 11). Through these studies, a general model of the associated signaling events has been established. Upon glucose depletion, Mig1p, a Cys2-His2 zinc finger DNA-binding protein is phosphorylated and localized in the cytoplasm. However, with the addition of exogenous glucose, Mig1p is dephosphorylated and imported into the nucleus, in a process that is facilitated by the nuclear transport receptor Msn5p (12, 13). Dephosphorylation is mediated by the inactivation of the Snf1-Snf4 protein kinase (14–16) and the activation of the Glc7-Reg1 protein phosphatase (17, 18). Once in the nucleus, Mig1p binds to the glucose-regulated promoters and recruits the general co-repressor complex Cyc8p-Tup1p to repress gene transcription (19, 20).

In contrast to the well characterized events that regulate signaling in transcriptional repression, our knowledge of the mechanisms that lead to the inactivation and degradation of gluconeogenic enzymes is not as well developed. FBPase has been the most extensively studied substrate for this process. After glucose-starved cells are replenished with fresh glucose, FBPase is phosphorylated, inactivated, and then degraded (5, 9, 21–23). To date, the site of FBPase degradation has been the subject of considerable controversy. For example, it has been reported that FBPase degradation is a proteasome-dependent process (24–29) where the proteolysis of FBPase depends on polyubiquitination and the function of the 26S proteasome. The N-terminal proline residue in FBPase is essential for proteasome degradation, because mutation of the proline residue inhibits FBPase degradation (25). However, mutation of the Ser-11 phosphorylation site did not affect the degradation of FBPase in the proteasome, suggesting that phosphorylation is not required for this pathway of degradation (25). In contrast to these results, our laboratory and others have established that FBPase degradation depends upon the proper function of the vacuole (23, 30–41). In this vacuole-dependent degradation pathway, FBPase is first imported into intermediate transport vesicles known as Vid vesicles (39). This import requires the action of a number of proteins, including Ssa2p (32), cyclophilin A (33), and Vid22p (34). The loaded Vid vesicles then traffic to the vacuole and deliver FBPase for degradation, via a process that resembles heterotypic vesicle fusion. This event is dependent upon Vid24p, a small GTPase, Ypt7p, and v-SNARE proteins on Vid vesicles, whereas the t-SNARE Vam3p is required on the vacuole (36). The type I phosphatase Glic7p and its regulator Reg1p have also been shown to be required for the fusion of Vid vesicles with the vacuole (41).

In addition to FBPase, the cytosolic enzyme malate dehydrogenase (MDH2) is subjected to rapid degradation when glucose-starved cells are replenished with fresh glucose (42, 43). Mu-
Distinct Proteolytic Pathways for Gluconeogenic Enzymes

This signaling pathway is required for the phosphorylation of FBPase and MDH2. Post-translational events downstream to increase transformation efficiency. A BLAST search was performed on the selected reverse primer sequences to ensure their specificity. The PCR primer sequences used in this study are listed in Table II. The PCR mix contained 100 pmol of each primer, 2 mM MgCl₂, 250 μM dNTPs, and 5 units of Taq polymerase (Qiagen). The thermo-

tageneic studies have shown that the first 12 amino acids at the N terminus in MDH2 (ΔnMDH2) are essential for degradation. More recently, an interaction between MDH2 and FBPase has been demonstrated using the yeast two-hybrid assay and surface plasmon resonance analysis (44). Again, the N-terminal 12 amino acids are important for MDH2-FBPase interaction, because FBPase interacted with ΔnMDH2 with a much lower affinity. These results suggest that the first 12 amino acids of MDH2 mediate both interactions and the proper targeting of MDH2 for degradation.

In the current study, we attempted to resolve three questions: 1) Is FBPase degraded by both the proteasome and vacuole and if so, what conditions determine the site of FBPase degradation?; 2) Is FBPase degradation unique, or do other cytosolic enzymes share the same degradation characteristics?; and 3) If FBPase can be targeted and degraded by distinct proteolytic pathways, what are the signals that control this process? To address these questions, we investigated whether variations in growth conditions could contribute to differences in FBPase degradation. We showed that the degradation of FBPase progressively shifted from a non-vacuolar proteolytic pathway to a vacuolar pathway when cells were starved for longer periods of time in glucose-limited medium. We also showed that MDH2 exhibited the same degradation characteristics as FBPase in that it was degraded in the non-vacuolar pathway when cells were starved for the short term, but it was degraded in the vacuole when cells were starved for longer periods of time. The N-terminal proline was required for the degradation of FBPase and MDH2, whether cells were subjected to short term or long term starvation conditions. However, the cAMP-dependent signaling pathway was exclusively involved in the vacuolar degradation of FBPase and MDH2. This signaling pathway is required for the phosphorylation of FBPase as well as the fusion of Vd vesicles with the vacuole. These results suggest that the cAMP signaling pathway plays multiple roles in the vacuole-dependent degradation of FBPase in response to glucose.

EXPERIMENTAL PROCEDURES

Yeast Strains, Culture Conditions, and Reagents—Yeast strains used in this study are listed in Table I. Media utilized in experiments includedYPD (1% yeast extract (US Biological), 2% peptone (US Biological), and 2% glucose) and YPKG (1% yeast extract, 2% peptone, 1% potassium acetate, and 0.5% glucose). Yeast transformation was carried out using the lithium acetate/Tris EDTA protocol. For the induction of gluconeogenic enzymes, cells were grown at 30 °C in an environmental shaker (300 rpm) in YPKG for 1–3 days. Cell density was measured, and cells were shifted to YPD for 0–2 h to induce degradation of the gluconeogenic enzymes. For these experiments, aliquots of cells (1 A₆₀₀) were used for each time point. Rabbit anti-FBPase antibodies were produced as described previously (30, 31). The ΔpdcΔprp1Δprc1 strain (45) was obtained from Dr. A. Kornberg (Stanford University). Anti-MDH2 antibodies were obtained from Dr. McAlister-Henn (University of Texas, Houston, TX) (43). Horseradish peroxidase-conjugated anti-rabbit antibodies and horseradish peroxidase-conjugated anti-mouse antibodies were purchased from Amersham Biosciences. Western blots were visualized using the enhanced chemiluminescence (ECL) kit (PerkinElmer Life Sciences).

Construction of FBPase-GFP and MDH2-GFP—We utilized a PCR-based method developed by Longtine et al. (46). In brief, a set of bacterial plasmids bearing an enhanced GFP with selectable nutrient marker (HIS5+ or TRP1) was used. PCR primers were designed according to the published protocol with minor modifications (46). To maximize yeast transformation efficiency, the sequences corresponding to the genomic DNA in PCR primers were increased to 50 or 60 nucleotides in length. The location of reverse primers was also shifted further downstream to increase transformation efficiency. A BLAST search was performed on the selected reverse primer sequences to ensure their specificity. The PCR primer sequences used in this study are listed in Table II. The PCR mix contained 100 pmol of each primer, 2 mM MgCl₂, 250 μM dNTPs, and 5 units of Taq polymerase (Qiagen). The therm-
cycling conditions were as follows: after the initial denaturation at 94 °C for 5 min, 30–35 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. The PCR products were purified and concentrated using Promega PCR Cleanup kit (Madison, WI). Genomic integration was confirmed by PCR. To examine the localization of FBPase-GFP and MDH2-GFP, cells were grown in YPKG for 1 or 3 days. Cells were then shifted to YPD media for 0–2 h and examined by fluorescence microscopy using a Zeiss fluorescence microscope and Axiosview software.

**RESULTS**

**FBPase Is Degraded by Two Distinct Proteolytic Pathways**

We have shown previously that FBPase is degraded in the vacuole when glucose-starved cells are shifted to glucose-containing media (30–41). However, a proteasome-dependent degradation of this same enzyme has also been demonstrated by the Wolf group (24–29). The reason for this discrepancy is unclear, although different experimental conditions are used in the two laboratories. To address this issue, we investigated whether variations in growth conditions could result in different sites of FBPase degradation. In our previous studies, we used ∆pep4 cells to demonstrate that FBPase degradation occurs in the vacuole. The PEP4 gene encodes for proteasine A, a major proteinase of the vacuole (48, 49). However, other proteinases are still present in the vacuole of this mutant, leading to an incomplete block of FBPase degradation. Therefore, this partial inhibition of degradation in the ∆pep4 strain may have contributed to the controversy in this field. To overcome this problem, we used a mutant strain harboring triple deletions of the PEP4, PRB1, and PRC1 genes that encode proteasines A, B, and C, respectively. A defect in FBPase degradation in this mutant would suggest that degradation occurs in the vacuole, whereas normal FBPase degradation in this mutant would indicate an extravacuolar site of degradation.

In our initial experiments, we confirmed that FBPase degradation is dependent on vacuole proteinases when cells were grown under our previously established growth condition (YPKG for 48 h, Fig. 1A). Next, we examined whether variations in growth conditions may result in different degradation patterns. Wild type and ∆pep4∆prb1∆prc1 cells were grown in glucose-limited media for various periods of time, shifted to fresh glucose for 0–2 h, and examined for FBPase degradation. As expected, the control wild type cells degraded FBPase when cultured under all growth conditions (Fig. 1B). However, a progressive reduction of FBPase degradation was observed for the ∆pep4∆prb1∆prc1 mutant, depending on how long this strain had been cultured in glucose limited media (Fig. 1A). FBPase was rapidly degraded when this mutant strain was grown under low glucose conditions (YPKG) for 24 h. Following 36 h of starvation, however, FBPase degradation was somewhat delayed. The degradation of FBPase in the ∆pep4∆prb1Δprc1 cells was retarded following starvation for 48 h, whereas FBPase degradation in this mutant was somewhat delayed. The degradation of FBPase was further delayed following starvation for 72 h. Therefore, the rate of FBPase degradation in this ∆pep4∆prb1Δprc1 mutant is dependent on the duration of glucose starvation. These results suggest that FBPase is degraded predominately by a non-vacuolar pathway following 24 h of starvation. By contrast, a retarded degradation of FBPase in the ∆pep4∆prb1Δprc1 mutant following a 72-h starvation period suggests that FBPase is degraded primarily in the vacuole under this circumstance. Owing to these dramatic differences in FBPase degradation in 1-day- versus 3-day-
starved cells, all subsequent experiments were conducted using cells that were starved for these two time periods.

To further confirm that FBPase was degraded in the vacuole when 3-day-starved cells were shifted to glucose, we performed localization studies using GFP-tagged FBPase. The Δpep4Δprb1Δprc1 mutant exhibited high background fluorescence under both short term and long term starvation conditions and as such was not suitable for these studies. In contrast, the Δpep4 cell displayed low background fluorescence when transformed with an FBPase-GFP construct. When FBPase-GFP distribution was examined in the Δpep4 cell, a low but detectable fluorescence signal was observed in the cytosol at t = 0 in cells starved for 1 or 3 days (Fig. 2A). However, the FBPase-GFP signal disappeared rapidly following a shift of 1-day-starved cells to glucose, suggesting that FBPase-GFP was degraded in this cell. By contrast, a detectable signal of FBPase-GFP was present in the vacuole of Δpep4 cells that were starved for 3 days and then shifted to glucose (Fig. 2A). This study further confirmed our previous indirect immunofluorescence analysis (30), whereby FBPase was localized to the vacuole in Δpep4 cells in response to glucose.

The proteasome is a major cytosolic site of protein degradation (50, 51). Furthermore, it has been reported that FBPase is degraded in the proteasome (24–29). Therefore, we next determined whether the proteasome was responsible for FBPase degradation in 1-day-starved cells. For these experiments, we used a Δpdr5 cell that is sensitive to the effects of the proteasome inhibitor MG132 (52). This strain was chosen because this inhibitor is ineffective in most wild type strains. When 1-day-starved Δpdr5 cells were shifted to glucose in the absence of MG132, FBPase was rapidly degraded (Fig. 2B). However, the addition of MG132 to these same cells retarded FBPase degradation (Fig. 2B), suggesting that the proteasome plays some role in FBPase degradation, at least under 1-day starvation condition. Unfortunately, results for FBPase degradation in cells starved for 3 days were difficult to interpret. Under this condition, FBPase was expressed as a 25-kDa truncated form. Interestingly, when we examined a number of proteasome mutants, the majority of the FBPase was also present as a truncated form. Although the significance of this is not clear at the present time (see “Discussion”), truncated FBPase was stable following a glucose shift in these cells (Fig. 2B).

For the vacuolar-dependent pathway, FBPase is associated with intermediate transport vesicles (Vid vesicles) before being targeted to the vacuole (39). It has not been established, however, whether these vesicles play a role in proteasome-mediated degradation. Furthermore, if FBPase is targeted for degradation in two proteolytic pathways, FBPase distribution within cells may be different under different growth conditions. Wild type cells were starved for 1 or 3 days and then shifted to glucose for 30 min. Organelles were fractionated by differential centrifugation followed by separation on a sucrose density gradient. Using this protocol, we showed previously that soluble proteins were in lighter density fractions, whereas Vid vesicles were in heavier density fractions (36, 39). In 1-day-starved cells, most of the FBPase was found in light density fractions (Fig. 3A). By contrast, in 3-day-starved cells, FBPase exhibited a broader distribution with significant amounts of FBPase extending to heavier density fractions (Fig. 3A). The presence of multiple FBPase peaks may represent various stages of Vid vesicle maturation in wild type cells, and this may correlate with different functions of Vid vesicles. Regardless, our fractionation results indicate that FBPase distribution in response to glucose stimulation can change depending on starvation conditions.

To further establish that FBPase was degraded by two distinct proteolytic pathways, we used the Δypt7 and Δvam3 mutants. Both Ypt7p and Vam3p have been shown to play important roles in many known trafficking pathways leading to the vacuole (53–60). These include the sorting of carboxy peptidase Y to the vacuole (53), the targeting of autophagosomes to the vacuole, the cytosome to vacuole targeting pathway (54–56), and homotypic vacuole fusion (57–60). In addition, for the vacuole-dependent FBPase degradation pathway, the last step requires the participation of Ypt7p and Vam3p (36). Because Ypt7p and Vam3p are specific for the vacuolar trafficking pathways, we used these mutants to distinguish between the vacuolar versus non-vacuolar degradation of FBPase. The Δypt7 and Δvam3 mutants were glucose-starved for 1 or 3 days, shifted to fresh glucose, and examined for FBPase degradation (Fig. 3B). FBPase was degraded in both mutants following a 1-day star-
vation. However, FBPase degradation was impaired when these same mutants were grown under glucose starvation conditions for 3 days (Fig. 3B). These results support the conclusion that short term starvation leads to a non-vacuolar degradation of FBPase, whereas long term starvation leads to FBPase degradation in the vacuole.

MDH2 and FBPase Show Similar Degradation Characteristics—Glucose-induced degradation of MDH2 has been described previously (42, 43). However, whether this protein is degraded in the vacuole or proteasome has not been addressed. Therefore, we next determined whether MDH2 showed similar degradation characteristics as FBPase. Wild type cells and triple vacuolar mutants were glucose-starved for 1 and 3 days, shifted to glucose for various periods of time, and examined for the degradation of MDH2 (Fig. 4, A and B). Glucose addition induced a rapid degradation of MDH2 when the /H9004 pep4/H9004 prb1/H9004 prc1 cells were starved for 1 day (Fig. 4A). However, MDH2 degradation was impaired when this mutant was starved for 3 days (Fig. 4A). As expected, the control wild type cells degraded MDH2 whether cells were starved for 1 or 3 days (Fig. 4B).

To further confirm that MDH2 was targeted to the vacuole in 3-day-starved cells, we examined the distribution of MDH2-GFP in the /H9004 pep4/H9004 cell. MDH2-GFP was detected in the cytosol at t = 0 in the /H9004 pep4 cell starved for either 1 or 3 days (Fig. 4C). However, the MDH2-GFP signal disappeared when 1-day-starved cells were shifted to glucose for 2 h, suggesting that MDH2-GFP was degraded. In contrast, most of the fluorescence signal was found in the vacuole when 3-day-starved /H9004 pep4 cells were shifted to glucose for 2 h (Fig. 4C).

We next determined whether MDH2 exhibited similar localization characteristics as FBPase. To address this question, 1- and 3-day-starved cells were glucose-shifted and fractionated as described above and the fractions were blotted with MDH2 antibodies. MDH2 exhibited a similar distribution as FBPase (Fig. 5A). In 1-day-starved cells, the majority of MDH2 was distributed in lighter fractions. In 3-day-starved cells, however, high levels of this protein were found in lighter fractions, whereas smaller amounts of MDH2 were also detected in heavier fractions (Fig. 5A). Therefore, FBPase and MDH2 appeared to co-localize, whether they were targeted to the vacuolar or to the non-vacuolar degradation pathways.

If long term starved cells utilize Vid vesicles to deliver MDH2 to the vacuole for degradation, then the /H9004 ypt7/H9004 and /H9004 vam3/H9004 mutants should block the degradation of MDH2 in response to glucose. Fig. 5B shows that MDH2 degradation was normal for 1-day-starved /H9004 ypt7/H9004 and /H9004 vam3/H9004 cells. By contrast, the degradation of MDH2 was retarded in /H9004 ypt7/H9004 and /H9004 vam3/H9004 cells that were starved for 3 days (Fig. 5B). Therefore, MDH2 shows the same degradation characteristics as FBPase, in that a shift of short term starved cells to glucose leads to a non-vacuolar degradation of MDH2. By contrast, a shift of long term starved cells to glucose leads to a predominantly vacuolar-dependent degradation of MDH2.

Vacuolar and Proteasome Degradation Pathways Share Common Components—A number of VID and GID genes have been characterized. VID genes were originally isolated during a search for genes involved in the vacuolar degradation of FBPase (40), whereas GID genes were identified in a search for genes involved in the degradation of FBPase via the protea-
some (25). Interestingly, a recent genome-wide search has also identified a number of *VID* genes, including *VID24*, *VID28*, and *VID30*, that are also involved in the proteasomal-dependent degradation of FBPase (28). This suggests that common components exist for both proteolytic pathways. To confirm the dual roles of these *VID* and *GID* genes, we examined FBPase and MDH2 degradation in *vid* and *gid* mutants that were starved for 1 and 3 days. As is shown in Fig. 6, *Δgid2* and *Δgid8* mutants blocked FBPase degradation in 1-day-starved cells, a result that is consistent with earlier reports (28). However, FBPase degradation was also blocked in these strains when they were starved for 3 days. Likewise, the *Δvid24*, *Δvid28*, and *Δvid30* mutants blocked FBPase degradation under both starvation conditions (Fig. 6A). In a similar manner, these mutants were defective in MDH2 degradation, whether they were starved for 1 or 3 days (Fig. 6B). Thus, the proteasome and vacuole degradation pathways share common components for the degradation of two cargo proteins, FBPase and MDH2.

The N-terminal Proline Is Required for MDH2 and FBPase Degradation—The sequence required for the glucose-induced degradation of MDH2 has been mapped to the N-terminal 12 amino acids (42). Because this protein can be degraded in the vacuole or proteasome, we examined whether this sequence plays a role in one or both of these pathways. As is shown in Fig. 6, *Δvid24*, *Δvid28*, and *Δvid30* mutants blocked FBPase degradation in 1-day-starved cells, a result that is consistent with earlier reports (28). However, FBPase degradation was also blocked in these strains when they were starved for 3 days. Likewise, the *Δvid24*, *Δvid28*, and *Δvid30* mutants blocked FBPase degradation under both starvation conditions (Fig. 6A). In a similar manner, these mutants were defective in MDH2 degradation, whether they were starved for 1 or 3 days (Fig. 6B). Thus, the proteasome and vacuole degradation pathways share common components for the degradation of two cargo proteins, FBPase and MDH2.
proline residue is required for the vacuole degradation pathway has not been determined. We produced the P1S mutation and examined the degradation of FBPase in short term- and long term starved cells (Fig. 8B). The P1S mutation inhibited FBPase degradation in 1-day-starved cells. This P1S mutation also blocked FBPase degradation in 3-day-starved cells. Therefore, the same proline residue is required for both non-vacuolar and vacuolar proteolysis pathways.

FBPase is phosphorylated by the cAMP-dependent pathway when cells are shifted to glucose, and this phosphorylation has been mapped to the serine 11 residue (21, 22). Therefore, we determined whether phosphorylation of FBPase at serine 11 was required for its degradation. We produced an FBPase mutation that substitutes serine for alanine at position 11. This S11A mutation did not block FBPase degradation in 1-day-starved cells, suggesting that phosphorylation at this site is not required for FBPase degradation in short term starved cells (Fig. 8B). This is consistent with the report that the S11A mutation had no effect on FBPase degradation in the proteasome (27). The same mutants also blocked FBPase degradation in 3-day-starved cells (Fig. 8B). Therefore, phosphorylation at this site is not required for FBPase degradation in the vacuole.

Glucose Phosphorylation Is Required for FBPase Degradation—Because FBPase and MDH2 each can be degraded by two distinct proteolytic pathways, different signaling events may be required to mediate their degradation in the proteasome or the vacuole. For the proteasome-dependent pathway, glucose phosphorylation by hexokinases is required (27). The cAMP-dependent pathway also appears to be required for FBPase degradation, although this has been a matter of debate. For example, it was reported that the cAMP pathway is not involved in FBPase degradation in the proteasome (27). In contrast, a Ras2-dependent degradation of FBPase has been described (23). To clarify these issues, we next examined the role that these signaling pathways played in FBPase degradation under different growth conditions.

When glucose is added to cells, this compound is converted to glucose 6-phosphate via the actions of hexokinase 1, hexokinase 2, and glucokinase 1 (61–63). To determine the role that glucose phosphorylation plays in FBPase degradation, we examined various deletion mutants lacking these genes. The deletion of individual genes did not affect the degradation of FBPase or MDH2 (Fig. 9, A and B), suggesting that these genes have redundant functions. Consistent with this notion, a triple mutant harboring deletions of HXK1, HXK2, and GLK1 inhibited FBPase degradation in 1- and 3-day-starved cells (Fig. 9A). The same mutants also blocked MDH2 degradation in 1- and 3-day-starved cells (Fig. 9B). Therefore, glucose phosphorylation by these kinases is required for FBPase and MDH2 degradation in the proteasome and the vacuole.

The cAMP-dependent Signaling Pathway Is Involved in Vacuolar Degradation of FBPase and MDH2—The addition of glucose to cells that have been grown in media containing non-
FIG. 5. **Ypt7p** and **Vam3p** are required for MDH2 degradation when long term starved cells are shifted to glucose. **A**, wild type cells were starved for 1 and 3 days in YPKG and refed with fresh glucose for 30 min. Cell lysates were subjected to differential centrifugation and sucrose density gradients as described (36). The distribution of MDH2 was examined by Western blotting of the fractions from the sucrose gradient. **B**, Δ*vam3* and Δ*ypt7* cells were grown in YPKG for 1 and 3 days, shifted to glucose, and examined for MDH2 degradation.

**A.** FBPase

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**Fig. 6.** **Common components function in the vacuolar and proteasomal proteolytic pathways.** Wild type, Δ*gid2*, Δ*gid8*, Δ*vid24*, Δ*vid28*, and Δ*vid30* mutants were starved for 1 and 3 days and then shifted to fresh glucose. The degradation profiles of FBPase (A) and MDH2 (B) were examined. For MDH2 blots, note the presence of a minor, nonspecific band. This band was also present in blots from Δ*mdh2* cell lysates.
fermentable carbon sources causes a rapid increase in cAMP (64–66). Members of the cAMP-mediated pathway include the G protein-coupled receptor Gpr1p and the heterotrimeric G protein Gpa2p. These proteins stimulate cAMP synthesis by activating adenylate cyclases. The intracellular G proteins Ras1p and Ras2p also regulate the activity of adenylate cyclases (64–66). Increased cAMP then stimulates a cAMP-dependent kinase to phosphorylate target proteins. When FBPase and MDH2 degradation was examined in \( \text{H9004} \ gpr1 \), \( \text{H9004} \ gpa2 \), and \( \text{H9004} \ ras2 \) strains, they were degraded normally in 1-day-starved cells (Fig. 10, A and B). In contrast, when these cells were starved for 3 days, the degradation of FBPase and MDH2 was retarded (Fig. 10, A and B). Therefore, the cAMP-dependent signaling pathway appears to play some role in the degradation of FBPase in the vacuole, whereas it is not required for degradation that occurs outside the vacuole.

**Fig. 7.** The N-terminal domain is necessary for the degradation of MDH2. A, \( \Delta mdh2 \) cells were transformed with plasmids containing either wild type or various MDH2 truncations. Cells expressing wild type, \( \Delta1-4 \), \( \Delta5-8 \), \( \Delta9-11 \), and \( \Delta1-12 \) (\( \Delta \)) MDH2 were grown in YPKG for 1 (A) and 3 days (B). Cells were shifted to glucose for 0 and 2 h, and MDH2 degradation was examined.

**Fig. 8.** The N-terminal proline is required for the degradation of MDH2 and FBPase. A, site-directed mutagenesis was performed to produce the N-terminal proline to serine (P1S) substitution and a serine to alanine mutation at position 3 (S3A) in MDH2. The P1S and S3A mutants were confirmed by DNA sequencing and then transformed into \( \Delta mdh2 \) cells. Cells were grown in YPKG for 1 and 3 days. Cells were shifted to glucose and examined for MDH2 degradation. B, site-directed mutagenesis was used to produce the P1S and S1A mutations in FBPase as described under “Experimental Procedures.” These mutations were confirmed by DNA sequencing and transformed into the \( \Delta fbp1 \) cells. Cells were grown in YPKG for 1 and 3 days, and glucose was added for 2 h. FBPase degradation was examined.

Whereas the cAMP-dependent signaling pathway plays some role in the degradation of FBPase in the vacuole, it is not required for degradation that occurs outside the vacuole. The cAMP Pathway Mediates FBPase Phosphorylation and Vacuole Fusion—To more clearly define the role that members of the cAMP pathway play in the process of FBPase degradation, we attempted to determine the site of action of these proteins. Mutation of the S11 FBPase residue does not affect FBPase degradation (Fig. 8B), indicating that...
phosphorylation at this site is not required for the glucose-induced degradation of this protein. However, whether other residues can be phosphorylated by protein kinase A has not been determined. To address this question, we determined whether the S11A FBPase mutant protein was phosphorylated in response to glucose. Cells expressing wild type and S11A FBPase were glucose-shifted, and FBPase phosphorylation was examined (Fig. 11A). For these experiments, similar amounts of FBPase were precipitated as detected by Western blotting with anti-FBPase antibodies (not shown). In wild type cells, FBPase phosphorylation was low at \( t = 0 \), and increased at \( t = 30 \) min. This phosphorylation was severely reduced in the protein kinase A mutant that harbors mutations in the \( \text{TPK1, TPK2, TPK3, and BCY1} \) genes, suggesting that phosphorylation is dependent on protein kinase A. Interesting, the S11A-FBPase was phosphorylated in response to glucose at \( t = 30 \) min. Therefore, serine 11 is apparently not the only phosphorylation site in the FBPase protein.

Next, we examined the distribution of FBPase in mutants that were shifted to glucose for 30 min. If the cAMP pathway is involved in FBPase import into Vid vesicles, then FBPase should accumulate in the cytosol in these mutants. However, if this signaling pathway is involved in Vid vesicle/vacuole fusion, FBPase should accumulate in Vid vesicle fractions. When FBPase distribution was examined, a significant portion of FBPase was detected in the Vid vesicle pellet fraction in the \( \Delta \text{ras2} \) mutant as well as in the cytosolic fraction (Fig. 11B). The distribution was similar to that seen in the \( \Delta \text{id24} \) mutant that blocks fusion of Vid vesicles with the vacuole (36). Similar FBPase distribution patterns were obtained with the \( \Delta \text{gpr1} \) and \( \Delta \text{gpa2} \) mutants (not shown). Because FBPase accumulated in Vid vesicle fractions, these results suggest that the cAMP signaling pathway is involved in the downstream fusion of Vid vesicles with the vacuole.

To verify a role for cAMP signaling in Vid vesicle/vacuole fusion, we utilized an in vitro fusion assay previously developed.
in our laboratory (36). For these experiments, Vid vesicles, vacuoles, and cytosol were isolated from wild type and \( /H9004 \) ras2 mutant cells that had been shifted to glucose for 30 min (36). Mutant and wild type components were combined as indicated in Fig. 11C, and fusion was measured in terms of the activation of alkaline phosphatase. When cytosol, vesicles, or vacuoles were isolated from the \( /H9004 \) ras2 mutant, alkaline phosphatase activity was low (Fig. 11C), indicating that one or all of these components were defective. To identify the site of this defect, various combinations of mutant and wild type components were tested. Alkaline phosphatase activity was low when mutant vesicles were mixed with wild type vacuoles and cytosol. Likewise, when \( /H9004 \) ras2 vacuoles were combined with wild type vesicles and cytosol, alkaline phosphatase activity was also low. In contrast, when the cytosol from \( /H9004 \) ras2 mutant was used, the fusion activity was high. Thus, the \( /H9004 \) ras2 mutant contained defective Vid vesicles and defective vacuoles, but normal cytosol. Taken together, these results suggest that the cAMP signaling pathway is required for multiple events in the FBPase degradation process. These include the phosphorylation of FBPase, as well as the fusion of Vid vesicles with the vacuole. Furthermore, both the vesicles and the vacuoles appear to be the site of action for this signaling pathway.

**DISCUSSION**

The site of FBPase degradation in response to glucose has been a matter of debate. FBPase has been reported to be ubiquitinated and degraded in the proteasome (24–29). However, FBPase also appears to be degraded in the vacuole via a Vid vesicle-dependent process (30–41). In addition to these different conclusions regarding the site of degradation, conflicting results were reported concerning whether the cAMP signaling pathway is involved in FBPase degradation. One report has indicated that this signaling pathway is not required for FBPase degradation (27). However, a Ras2-dependent increase in intracellular cAMP levels was also shown to be important for FBPase degradation (23). Because different growth conditions were used to induce FBPase expression in these laboratories, one explanation for this discrepancy is that growth conditions of cells determine the site of FBPase degradation. In this current study, we demonstrated that variations in growth conditions indeed led to different mechanisms of FBPase degradation. Here, we provided evidence showing that at least one of the factors that determines the fate of FBPase is the duration of the starvation condition, even in cells that were grown in the same glucose limited culture media. The addition of glucose to cells that underwent short term starvation (1d) resulted in a non-vacuolar degradation of FBPase. By contrast, the addition of glucose to long term (3-day)-starved cells led to the degradation of FBPase in the vacuole.

For 1-day-starved cells, FBPase is most likely degraded in the proteasome. Consistent with this observation, the addition of proteasome inhibitors to otherwise wild type cells inhibited FBPase degradation in 1-day-starved cells. However, caution should be taken when using proteasome inhibitors or proteasome mutants, because they may cause indirect effects. In our studies, long term starvation in several proteasome mutants led to the expression of FBPase as a 25-kDa truncated form. Interestingly, this truncated form of FBPase was not degraded.
in these proteasome mutants following glucose refeeding (not shown). In a similar manner, the truncated form of FBPAse was not degraded when 3-day-starved Δprp5 cells were transferred to glucose in the presence of the proteasome inhibitor. Although the reason for this inhibition is not clear at the present time, one explanation is that the truncated FBPAse lacks critical domains necessary for degradation to occur. As a result, this protein remains stable. Another possibility is that the use of proteasome mutants or proteasome inhibitors changed the ubiquitin pool within cells. A reduction in free ubiquitin levels can potentially impair the functions of conjugating enzymes to ubiquitinate substrate proteins. In our previous studies, we showed that at least one of the ubiquitin-conjugating enzymes, Ubc1p, is required for Vid vesicle formation and function in the vacuole pathway (38). Therefore, when the free ubiquitin pool is reduced, Ubc1p activity may be affected. This can lead to an FBPAse import defect and a subsequent degradation defect.

Owing to the limitations of experiments with proteasome mutants or inhibitors, we believed that more compelling evidence can be obtained through the examination of FBPAse degradation in Δpep4Δprp1Δprc1 mutants deficient in major vacuole proteasines. When these mutant strains were grown under long term starvation conditions, FBPAse degradation in response to glucose was severely retarded, suggesting that FBPAse degradation occurs in the vacuole. Furthermore, FBPAse-GFP was found in the vacuole when 3-day-starved Δpep4 cells were shifted to glucose, indicating that FBPAse was targeted to the vacuole under this condition. Additional support for the vacuole-dependent degradation of FBPAse came from studies with the Δyp77 and Δycm3 mutants. These strains also blocked FBPAse degradation in 3-day-starved cells, but not in 1-day-starved cells. Based upon these observations, we conclude that long term starvation leads to the degradation of FBPAse in the vacuole.

In this study, we identified MDH2 as another substrate for both degradation pathways. Both FBPAse and MDH2 were degraded in the vacuole in long term starved cells. Likewise, they were degraded in the non-vacuolar pathway in short term starved cells. It is intriguing that both proteins require the N-terminal proline residue for the vacuolar and the non-vacuolar degradation pathways. Accordingly, the PIS mutation on both proteins inhibited their degradation in short term as well as long term starved cells. For the proteasomal degradation pathway, the N-terminal proline is necessary for the ubiquitination of FBPAse (25). However, the vacuolar degradation pathway is unlikely to utilize this system, because FBPAse ubiquitination was not observed under the vacuolar degradation conditions (38). FBPAse is known to interact with MDH2 under glucose starvation conditions (44). Therefore, we determined whether the degradation defect seen in the PIS-FBPAse and PIS-MDH2 mutants resulted from a reduction in their interactions. We compared the interactions between the PIS-FBPAse with the wild type MDH2 to the wild type counterparts. Unfortunately, we did not observe any reduction in the FBPAse-MDH2 interaction when the PIS-FBPAse mutant was used. In a similar manner, the interaction between PIS-MDH2 and wild type FBPAse was not reduced. Furthermore, the interactions remained the same, before or after glucose shift (not shown). Therefore, the N-terminal proline is not required for the FBPAse-MDH2 interaction. We suspect that the N-terminal proline serves as a determinant by binding to an as yet unidentified factor or factors. If this scenario is true, it will be important to identify these factors and to determine how they function in the vacuolar pathway.

Finally, we showed that signaling pathways play important roles in determining whether cells degrade FBPAse and MDH2 in the vacuole or in the proteasome. Phosphorylation of glucose by hexokinases and glucokinase is required for FBPAse and MDH2 degradation, not only for the vacuole pathway, but also for the proteasome pathway. In contrast, the cAMP-dependent signaling pathway is exclusively involved in vacuole-dependent degradation. Deletions of components of the cAMP signaling pathway, including GPA2, GPR1, and RAS2, all inhibited FBPAse and MDH2 degradation in short term starved cells. Although FBPAse is known to be phosphorylated on serine 11 residue (21, 22), the S11A mutation in FBPAse did not affect degradation in short term or long term starved cells. Furthermore, when this mutant was examined, it was phosphorylated in response to glucose at a level similar to wild type FBPAse. Therefore, serine 11 is not the only site for FBPAse phosphorylation.

In the Δras2 mutant, a significant amount of FBPAse was associated with Vid vesicles, suggesting that Ras2 is involved in the fusion of Vid vesicles with the vacuole. This is consistent with the results that both vesicles and vacuole were defective in fusion in the Δras2 mutant. Our results suggest that the cAMP signaling pathway plays multiple roles in the vacuole degradation pathway. It is required for the phosphorylation of FBPAse, but the serine 11 residue is not the only target for phosphorylation. This signaling pathway is also required for the proper function of Vid vesicles and the vacuole. Further experiments will be needed to identify the downstream effectors regulated by the cAMP signaling pathway and to identify factors that recognize the N-terminal proline residue.

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Distinct Proteolytic Pathways for Gluconeogenic Enzymes

Degradation of the Gluconeogenic Enzymes Fructose-1,6-bisphosphatase and Malate Dehydrogenase Is Mediated by Distinct Proteolytic Pathways and Signaling Events
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