Fructose-1,6-bisphosphatase (FBPase), the key enzyme in gluconeogenesis in the yeast *Saccharomyces cerevisiae*, is induced when cells are grown in medium containing poor carbon sources. FBPase is targeted from the cytosol to the vacuole for degradation when glucose-starved yeast cells are replenished with fresh glucose. In this study, we report the reconstitution of the glucose-induced import of FBPase into the vacuole in semi-intact yeast cells using radiolabeled FBPase, an ATP regenerating system and cytosol. The import of FBPase was defined as the fraction of the FBPase that was sequestered inside a membrane-sealed compartment. FBPase import requires ATP hydrolysis and is stimulated by cytosolic proteins. Furthermore, the import of FBPase is a saturable process. FBPase import is low in the glucose-starved cells and is stimulated in the glucose-replenished cells. FBPase accumulates to a higher level in the *pep4* cell, suggesting that FBPase is targeted to the vacuole for degradation. Indirect immunofluorescence microscopy studies demonstrate that the import of FBPase is localized to the vacuole in the permeabilized cells. Thus, the glucose-induced targeting of FBPase into the vacuole can be reproduced in our *in vitro* system.

The vacuole of the yeast *Saccharomyces cerevisiae* is an acidic compartment surrounded by a lipid bilayer. Vacuoles contain a variety of soluble and membrane-bound proteolytic enzymes and are considered to be equivalent to the lysosome of higher eukaryotes (1). The proteolytic processes in the vacuole play an important role when the cells are under nutritional stress. The vacuole is also responsible for the degradation of overexpressed proteins, normal cellular proteins, and some abnormal proteins (2).

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. FBPase is rapidly degraded when fresh glucose is added to the glucose-starved yeast cells (3). FBPase is targeted from the cytosol to the vacuole for degradation in response to glucose (4, 5). The degradation of FBPase is dependent on the *PEP4* gene encoding proteinase A, which is necessary for the activation of proteinase B and C (6). As a result, the deletion of the *PEP4* gene reduces the vacuolar proteolytic activity to 30% that of the wild type level (6). In the *pep4* deletion strain, FBPase is found in the vacuole when glucose-starved cells are transferred to fresh glucose (4, 7). In addition to FBPase, several other proteins and organelles are also delivered to the vacuole for degradation when cells are shifted to the glucose medium. They include formate dehydrogenase (8), plasma membrane proteins such as the maltose transporter (9) and the galactose transporter (7), and peroxisomes (7, 8, 10).

Most vacuole resident proteins pass through early parts of the secretory pathway en route to the vacuole (2, 6, 11, 12). For instance, carboxypeptidase Y (CPY) is synthesized and processed sequentially in the endoplasmic reticulum and the Golgi. CPY is sorted in the late Golgi by the CPY receptor and is delivered to the vacuole through the prevacuolar/endosomal compartment (13, 14). In contrast, targeting of vacuolar aminopeptidase I and α-mannosidase to the vacuole is independent of the secretory pathway (15–17). Plasma membrane proteins can be internalized by endocytosis and transported through endosomes to the vacuole for degradation (9, 18–22). Other organelles such as peroxisomes or mitochondria can be engulfed by the vacuole by autophagy (7, 8).

Biochemical reconstitution of protein sorting has been successfully applied to study the transport of proteins through the secretory pathway including translocation into the endoplasmic reticulum (23, 24), endoplasmic reticulum to Golgi transport (25–29), and intra-Golgi (30, 31) and late Golgi to vacuole transport (32, 33). Protein import into the nucleus (34), mitochondria (35), and peroxisomes (36) have also been reproduced *in vitro*. Homotypic fusion of vacuoles *in vitro* has been used to study processes required for vacuolar inheritance (37, 38). In addition, targeting of aminopeptidase I from the cytoplasm to the vacuole has also been reconstituted (39). Degradation of cytosolic proteins by isolated lysosomes has been documented in mammalian cells (40–42). However, targeting of cytosolic proteins into the yeast vacuole has not been reconstituted *in vitro*.

To investigate the pathway of FBPase degradation, we reconstituted *in vitro* FBPase targeting into the vacuole using permeabilized yeast cells incubated with radiolabeled FBPase, an ATP regenerating system, and cytosolic proteins. The import is a saturable process and is stimulated by glucose. Import of radiolabeled FBPase into semi-intact cells is competed by excess unlabeled FBPase but not a control protein. The import of FBPase accumulates to a higher level in the *pep4* cell, suggesting that FBPase is targeted to the vacuole for degradation. Immunofluorescence microscopic studies show that the imported FBPase is localized to the vacuole. Thus, this *in vitro* system reconstitutes glucose-induced targeting of FBPase into the vacuole and provides a functional assay to identify molecules required for the FBPase targeting and degradation pathway.
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EXPERIMENTAL PROCEDURES

Media and Yeast Strains—S. cerevisiae strains used in this study were HLY193 (Matα his3-D200 ura3-52 leu2, 3-112 trp1 FYPL1::LEU2) and HLY205 (Matα his3-D200 ura3-52 leu2, 3-112 trp1 FYPL1::LEU2 PEP4::TRP1). All the chemicals, unless otherwise noted, were obtained from Sigma or ICN Biochemicals Inc. YPD was a complete medium (10 g/liter Bacto yeast extract, 20 g/liter Bacto peptone; Difco) supplemented with 20 g/liter dextrose (Fisher) and was used to induce FBPase degradation. Synthetic minimal medium contained 6.7 g/liter yeast 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. YPKG was the FBPase-inducing medium and contained 10 g/liter Bacto yeast extract, 20 g/liter Bacto peptone, 10 g/liter potassium acetate, and 5 g/liter magnesium acetate. 3S-Protein labeling mix ([35S]methionine) was obtained from NEN Life Science Products. The FBPase expression plasmid (AU125) was a gift from Dr. David T. Rogers (43). Rabbit anti-FBPase and rabbit anti-CPY polyclonal antibodies were raised by Berkeley Antibody Company using purified FBPase and CPY (Sigma).

**Purification of Fructose-1,6-bisphosphatase**—The FBPase expression plasmid AU125 (45) was introduced into S. cerevisiae strains by lithium acetate transformation (44). The transformed cells overexpressing the FBPase gene were grown overnight at 30 °C in the uracil- and methionine-free synthetic medium (without ammonium sulfate) containing 20 g/liter dextrose and 10 mM/liter of 35S-methionine. The radiolabeled FBPase was purified according to the procedure described by Rittenhouse et al. (45), except that P11 column was omitted. FBPase was the major protein band (~80% that of the total proteins) on the Coomassie Blue-stained gels. The protein concentrations from these preparations were ~5–10 mg/ml as determined by Bio-Rad D, protein assay kit (Bio-Rad). The specific activity was 4–7 × 106 cpm/μg of the protein. The enzyme was stored at −70 °C in small aliquots. For antibody production, the FBPase-overexpressing cells were grown in synthetic minimal medium overnight, and FBPase was purified as described (45).

**Preparation of Semi-intact (Permeabilized) Cells**—S. cerevisiae cells (20 g/liter dextrose culture) were grown in YPD for 2 days at 30 °C. Cells were harvested and resuspended in YPD and incubated at 30 °C for different periods of time. At the end of the incubation, cells were chilled by adding ice water, collected by centrifugation (1,500 × g, 5 min) at 4 °C, and washed once with water. Spheroplasts were prepared as described previously (4) and collected by centrifugation (15,000 × g, 2 min). Spheroplasts were washed with ice-cold sorbitol buffer (1.2 M sorbitol, 0.2 mg/ml creatine phosphokinase, and 40 mM creatine phosphate), centrifuged at 13,000 × g for 10 min at 4 °C. The precipitate was washed once with ice-cold acetone and resuspended in 200 µl of SDS loading buffer. The proteins (~15 µg) were then resolved on SDS-PAGE. Gels were stained with Coomassie Blue, dried, and analyzed by a Fuji FUJIX BAS 100 Bioimaging analyzer (Fuji Medical Systems). Alternatively, proteins were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell) for immunoblotting. FBPase and CPY were detected by anti-FBPase and anti-CPY antibodies using the enhanced chemiluminescence (ECL) immunoblotting procedure (46). The import of FBPase was defined as the fraction of 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 the detergent. In general, 20–40% of import could be achieved in the in vitro system.

**Indirect Immunofluorescence**—The semi-intact cells were treated with proteinase K at the end of import reactions. Semi-intact cells were collected by centrifugation (13,000 × g, 20–30 s) and resuspended in 1 ml ice-cold sorbitol buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.3 M sorbitol, 1 mM EDTA). Cells were fixed and stained with anti-FBPase antibody as described previously (4) with the following modifications. Cells were fixed with 7% formaldehyde for 40 min at room temperature, washed twice with 0.5 ml of 1.2 M sorbitol and incubated with pre-absorbed rabbit anti-FBPase antibodies (1:20 dilution) overnight and with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies for 1 h. Cells were visualized by Nomarski optics, and FBPase was detected by immunofluorescence using Zeiss Axioskop microscope. Images were analyzed with the Northern Exposure and Adobe Photoshop softwares.

**RESULTS**

**The Import System**—To reconstitute FBPase import into the vacuole, we prepared semi-intact cells from the pep4 and fbp1 deletion strain. The deletion of the endogenous FBPase gene (FBP1) was necessary to introduce radiolabeled FBPase exogenously. The pep4 deletion strain reduces the vacuolar proteolytic activity to 30% that of the wild type level and allowed FBPase to accumulate to a higher level and hence facilitated the detection of the import process. The overall strategy to reconstitute in vitro import of FBPase is illustrated in Fig. 1. The semi-intact cells, unless otherwise noted, were prepared from the Δpep4 Δfbp1 (HLY205) yeast cells that were grown to induce FBPase and then transferred to medium containing fresh glucose for 20 min. Semi-intact cells were prepared from the double deletion strain by removing cell walls to produce spheroplasts, followed by slow freeze and thaw and treating with hypotonic buffer. This releases soluble proteins and small molecules into the medium and still preserves the integrity of most organelles in a functional state (25). Using this approach, we introduced purified, radiolabeled FBPase into semi-intact cells and followed the fate of radiolabeled FBPase in the permeabilized cells.

**FBPase Is Imported into a Membrane-sealed Compartment**—We first determined whether FBPase was imported into a proteinase K-resistant compartment in the permeabilized cells in vitro. In the standard import conditions, the total FBPase, which consisted of both imported and nonimported FBPase, migrated at 38 kDa on the SDS-PAGE gels (Fig. 2A, lane 1). To distinguish the FBPase that was imported into a membrane-sealed compartment from the FBPase that was not, we added protease K to digest the free FBPase after the import had occurred. This treatment produced two species of FBPase: the full-length 38-kDa FBPase and the smaller sized 30-kDa FBPase (Fig. 2A, lane 3). The 38-kDa FBPase represented the FBPase that was protected in a membrane-sealed compartment, since it was sensitive to proteinase K digestion when Triton X-100 was added (Fig. 2A, lane 4). By contrast, the
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Figure 1. Outline of the strategy to reconstitute FBPase import into the vacuole in permeabilized cells. To facilitate measurement of FBPase import, semi-intact cells and cytosol were prepared from a pep4 and fbp1 deletion strain that had been shifted to glucose for 20 min. Semi-intact cells were incubated with cytosol, an ATP regenerating system and 35S-FBPase (*). At the end of the reactions, proteinase K treatment under this condition (Fig. 2A, lane 2) 98% of the full-length FBPase was converted to 30 kDa by proteinase K treatment in this 35S-FBPase, cytosol, and an ATP regenerating system for 20 min, and increasing amounts of proteinase K were added after the reactions. Fig. 2C shows that in the absence of Triton X-100, approximately 30–40% of the total FBPase was resistant to proteinase K over the concentration ranges from 0.1 to 1.6 mg/ml. At a concentration of 0.4 mg/ml proteinase K, essentially all the FBPase was digested when Triton X-100 was added, approximately 30–40% of the total FBPase was resistant to proteinase K over the concentration ranges from 0.1 to 1.6 mg/ml. Proteinase K was sufficient to digest most of the free FBPase and unravel the fraction of FBPase that was protected in a membrane-sealed compartment, we added 8.0 mg/ml proteinase K at the end of our in vitro incubations for all the subsequent analysis.

FBPase Import Is a Saturable Process—In the wild type cell harboring a chromosomal copy of the FBP1 gene, FBPase is degraded with a half-life of 30 min in response to glucose. The degradation of FBPase is retarded if the cells overexpress the FBP1 gene; the half-life of FBPase degradation increases to 60–90 min in such cells (4). The dose-dependent increase in the FBPase degradation half-lives suggests that FBPase targeting to the vacuole is a saturable process. We tested whether the import of FBPase was a saturable process in the reconstituted system by incubating semi-intact cells with increasing amounts of radiolabeled FBPase in the absence or presence of an ATP regenerating system and cytosol. As shown in Fig. 2A, FBPase import was minimal in the absence of ATP, whether or not cytosol was added to the medium. FBPase import increased when ATP was added in the absence of cytosol. FBPase import was the highest when both ATP and cytosol were present in the incubation medium. Quantitation of the results obtained in the presence of ATP and cytosol showed that the import of FBPase increased when the amounts of FBPase were raised from 2.2 to 11 μg (Fig. 3B). However, when the amount of the exogenous FBPase was increased to 22 μg, the amount of FBPase that was
We titrated the concentrations of cytosolic proteins and determined the effects of cytosolic proteins in FBPase import in the in vitro system. As shown in Fig. 4A, the import was very low in the absence of both ATP and cytosol, and the import of radiolabeled FBPase was quantitated by phosphorimaging. 100% is defined as FBPase import in the absence of unlabeled proteins.

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FIG. 3. FBPase import is a saturable process. Various amounts of $^{35}$S-FBPase (2.2–22 μg) were added to the standard assay, and FBPase import was measured as described in Fig. 2. A, proteins were resolved by SDS-PAGE, and the imported FBPase was analyzed by ECL immunoblotting. B, the same samples were quantitated by phosphorimaging. The level of FBPase in the absence of ATP and cytosol was used as a background and was subtracted from each reaction. The open circle represents FBPase import in the presence of both ATP and cytosol, the open square represents FBPase import in the presence of ATP without cytosol, and the open triangle represents the import in the presence of both ATP and cytosol. The data is the average of duplicate samples. C, standard FBPase import reactions were carried out as described. The radiolabeled FBPase (11 μg) was incubated with 10 or 30 μg of unlabeled FBPase (filled circles) or bovine serum albumin (filled triangles), and the import of radiolabeled FBPase was quantitated by phosphorimaging. 100% is defined as FBPase import in the absence of unlabeled proteins.

FIG. 2. Time course of vacuole stability and FBPase import. A standard FBPase import assay was performed. The reaction mixture (100 μl) contained 3 A$_{260}$ units of semi-intact cells (HLY205), 0.5 mg/ml cytosolic proteins, an ATP regenerating system (0.5 mM ATP, 0.2 mg/ml creatine phosphokinase, and 40 mM creatine phosphate), and 11 μg of $^{35}$S-FBPase in the import buffer. The mixture was incubated at 30 °C for 20 min. At the end of incubation, 50 μl of proteinase K solution (2.4 mg/ml proteinase K, 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.3 M sorbitol, 1 mM EDTA with or without 6% Triton X-100) was added, and the mixture was incubated at room temperature for 15 min. The reaction was terminated by adding 1 ml of 15% w/v trichloroacetic acid to the mixture, and the reaction was neutralized by 0.1 M sodium hydroxide. After addition of 1 ml of 15% w/v trichloroacetic acid, the samples were centrifuged, and the supernatants were analyzed for proteinase K and FBPase activity. The import was measured as described in Fig. 2. A, lane 1, FBPase import in complete reaction mixture, without proteinase K treatment. Lane 2, FBPase import in the presence of 2% Triton X-100. Lane 3, FBPase import in the absence of Triton X-100. Lane 4, after FBPase import, proteinase K was added in the absence of Triton X-100. Lane 5, after FBPase import, proteinase K was added in the presence of Triton X-100. Lane 6, after FBPase import, proteinase K was added in the presence of 2% Triton X-100. Lane 7, after FBPase import, proteinase K was added in the presence of 2% Triton X-100, and FBPase import was measured as described in Fig. 2. A, lane 1. B, time course of FBPase import. Lane 1, FBPase import in the complete reaction mixture (as described in Fig. 2A, lane 1) and the amounts of p2-CPY were followed from 0 to 60 min at 30 °C. C, FBPase import in the absence of both ATP and cytosol was measured as described in Fig. 2A, lane 1. D, the same samples were quantitated by phosphorimaging.
Glucose-stimulated proteolytic degradation in the vacuole. Glucose may regulate the production of important factor(s) that are required for importing FBPase into the vacuole. We determined whether the import would be regulated by glucose. Cells were transferred to glucose for 0–60 min, and aliquots were transferred to YPD medium for 0–60 min. Semi-intact cells were prepared for the FBPase import assay. The kinetics of FBPase import was followed for 0–60 min.

**FBPase Accumulates at a Higher Level in the pep4 Cell—** FBPase import requires cytosol and ATP. A, different amounts of cytosolic proteins (0, 0.5, and 1 mg/ml) were added to the import reaction mixtures in the presence or absence of 0.5 mM ATP. Samples were processed and analyzed as described in Fig. 2. The open bar shows FBPase import without ATP, and the hatched bar shows the import in the presence of 0.5 mM ATP. The histogram is the average of three experiments. B, FBPase import was carried out as described in Fig. 2 with 0–1 mM ATP added in the presence of 0.5 mg/ml cytosol. The nonhydrolyzable ATPγS was added at 0, 10, or 50 μM to the complete mixture containing 0.5 mM ATP and 0.5 mg/ml of cytosol.

0.5 mM ATP in the absence of cytosol increased FBPase import. The maximal import was found when 0.5 mM ATP was combined with cytosol at the concentration of 0.5 mg/ml. No further increase was seen when the concentration of cytosol was raised to 1 mg/ml. Therefore, cytosol has a synergistic effect on FBPase import and stimulates the import 2–3-folds when combined with ATP in the import reactions.

We also determined the optimal concentrations of ATP in the presence of 0.5 mg/ml cytosol in the reconstituted system (Fig. 4B). As we have already shown, FBPase import did not occur in the absence of ATP. However, the addition of ATP stimulated FBPase import in a dose-dependent manner (Fig. 4B). The import increased at 0.2 mM ATP and reached a maximum at 0.5 or 1 mM ATP. When the nonhydrolyzable ATP analogue, ATPγS, was added to compete with ATP, FBPase import was inhibited (Fig. 4B). These results indicate that FBPase import requires the hydrolysis of ATP.

**Glucose Stimulates FBPase Import—** FBPase is subjected to glucose-stimulated proteolytic degradation in the vacuole. Glucose may regulate the production of important factor(s) that are required for importing FBPase into the vacuole. We determined whether the import would be regulated by glucose. Cells were transferred to glucose for 0–60 min and were permeabilized. FBPase import was assayed in semi-intact cells prepared from glucose-starved (t = 0 min) or glucose-replenished (t = 10 to t = 60 min) cells. As shown in Fig. 5, FBPase import was low in the glucose-starved cells (Fig. 5A, t = 0 min). The import was higher in cells that were shifted to glucose for 20–30 min (Fig. 5, C and D). FBPase import was reduced in cells that had been shifted to glucose for 45 or 60 min (Fig. 5, E and F). These results suggest that the effect of glucose on FBPase import is at its maximum in cells that have been transferred to the glucose-rich medium for 20–30 min.

**FBPase Accumulates at a Higher Level in the pep4 Cell—** To examine whether FBPase was imported and degraded in the vacuole, we compared FBPase import in a wild type strain to that in the pep4 deletion strain. Both strains had been transferred to glucose for 20 min, since the highest import was observed under this condition in the pep4 cell (Fig. 5). Accumulation of FBPase in the pep4 cell would indicate that FBPase was targeted into the vacuole for degradation. Fig. 6A shows that, in the wild type cell, the amounts of FBPase were low. When the semi-intact cells and cytosol were prepared from the pep4 cell, FBPase accumulated at higher levels (Fig. 6B). These results suggest that FBPase was targeted into the vacuole for degradation in the wild type cell; the accumulation of FBPase in the pep4 strain was a consequence of reduced proteolytic activity in the pep4 cell.

**Localization of the Imported FBPase—** We examined whether the imported FBPase was localized in the vacuole. Cells were proteinase K-treated and homogenized by gentle lysis. The vacuole was separated by Ficoll gradient separation. The distribution of CPY was determined by Western blotting with CPY antibodies. Using this method, the recovery of intact vacuole was less than 10%, suggesting that significant lysis of the vacuole occurred during the separation process (data not shown). A similar problem was encountered when the vacuole was isolated by sucrose density gradient. Because the recovery of intact vacuole was too low, no conclusion could be drawn as to whether FBPase was indeed targeted to the vacuole in the reconstituted system.

As an alternative, we used indirect immunofluorescence microscopy to examine the localization of the imported FBPase. At the import time of 0, 20, and 40 min, proteinase K was added to the semi-intact cells. Cells were then fixed with formaldehyde. Localization of FBPase was detected by probing with...
anti-FBPase antibodies followed by fluorescein isothiocyanate-conjugated secondary antibodies.

Consistent with the reports by several investigators (25, 34), the overall morphology of the semi-intact cells was significantly compromised. Fig. 7B, D, and F show representative images of the cells that contained recognizable vacuole as seen by Nomarski microscopy. When the Nomarski images were compared with fluorescence FBPase staining in these cells, localization of FBPase in the vacuole was observed at the import time of 20 and 40 min (Fig. 7, C and E). At the import time of 0 min, there was no detectable FBPase immunofluorescence (Fig. 7A). After import for 20 min, most of the FBPase staining was in small dots inside the region that corresponded to the vacuole as seen by Nomarski images (Fig. 7C). A similar pattern of FBPase staining in small dots inside the vacuole has been reported in vivo (4, 7). At the import time of 40 min, most of the FBPase staining was also observed in the vacuole. At this time point, the immunofluorescence intensity was reduced, suggesting that some of the imported FBPase had been degraded by vacuole proteinases (Fig. 7E).

The distribution of FBPase antigen in the vacuole was observed in both wild type and pep4 cells. However, the background staining of FBPase in the wild type strain was lower than that seen in the pep4 cell, which showed fluorescence even in the absence of the first antibodies for reasons that were unclear to us. Despite all the technical difficulties that we have encountered, the staining of FBPase in the vacuole in wild type cells at the import time of 20 and 40 min suggests that FBPase is targeted correctly to the vacuole and then degraded in the vacuole in the reconstituted system.

**DISCUSSION**

The *in vitro* reconstitution system is a powerful tool to dissect complicated biological events in cells. We developed an *in vitro* assay to study the targeting of a cytosolic protein FBPase to the yeast vacuole. We measured *in vitro* import of FBPase by incubating semi-intact cells with 35S-FBPase in the presence of an ATP regenerating system and cytosol. FBPase import is a saturable process (Fig. 3). Cells that have been transferred to glucose for 20 or 30 min and then permeabilized stimulate FBPase import to higher levels than the glucose-starved cells (Fig. 5). The import of FBPase requires ATP and cytosolic proteins (Fig. 4). It is inhibited by ATPγS, suggesting that ATP hydrolysis is important for FBPase import to occur. ATP may be required for the action of molecular chaperonins, targeting, or translocation of FBPase into organelle membranes or maintaining of the vacuole acidity. ATPγS may have much higher affinity than ATP for the ATP-binding factors. Once ATPγS is bound to such factors, it cannot be hydrolyzed and locks the ATP-binding proteins in a configuration that prevents further binding of ATP to the factors. This may explain why lower concentrations of ATPγS inhibit FBPase import in this *in vitro* system.

The degradation of FBPase requires the synthesis of new proteins, as FBPase degradation is inhibited by cycloheximide, an inhibitor of protein synthesis. The inhibition can only be observed if cycloheximide is added at the same time with glucose (4). Cycloheximide has no effect if this agent is added 20–30 min after glucose incubation, suggesting that important proteins for FBPase degradation are synthesized in the first 20–30 min of glucose readdition. Our *in vitro* system demonstrates that cells that have been shifted to glucose for 20–30 min and then permeabilized stimulate FBPase import to higher levels. These cells may contain a higher level of the factors induced by glucose. Cells that have been transferred to fresh glucose medium for 45 min or longer may have consumed these important factors and therefore import FBPase at lower levels.

We have shown in our previous study that the degradation of FBPase is dependent on the function of the PEP4 gene product *in vivo* (4). In this *in vitro* system, FBPase level is low in the wild type cell and accumulates at a higher level in the pep4 cell (Fig. 6). Since the pep4 strain contains reduced proteolytic activity, the higher level of FBPase in the pep4 cell is expected if FBPase is targeted to the vacuole and is degraded slowly by residual proteinases.

We have attempted to isolate the vacuole using different procedures. However, we could only recover less than 10% of intact vacuole after homogenization and gradient separation. This was observed whether cells were homogenized by osmotic lysis or with a Dounce homogenizer. The low recovery of intact vacuole occurred whether the vacuole was isolated by Ficoll floatation gradients or by sucrose density gradient. The semi-intact cells had been subjected to cycles of freeze and thaw and then treatments with hypotonic solution repeatedly before the import reaction. Since 20–40% FBPase was protected in the sequestered FBPase, presumably the vacuole, was likely to be intact during the import reaction. However, when cells were homogenized and further fractionated, the yield of intact vacuole was significantly decreased. Because of this problem, no
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In conclusion, it could be drawn as to whether the imported FBPase was indeed localized to the vacuole. Another factor that might contribute to the technical difficulties was the addition of excessive amounts of proteinase K at the end of the import reactions. Proteinase K was not a problem when samples were solubilized in SDS buffer, boiled immediately, and separated by SDS-PAGE electrophoresis. It might become a problem when cells were homogenized and fractionated. The large amounts of proteinase K might remain active and degrade cellular components nonselectively. As a result, the recovery of intact vacuole was significantly reduced.

This problem was partially circumvented by the immunofluorescence technique that required fixing of the semi-intact cells with formaldehyde, which also quenched the activity of proteinase K. When the FBPase staining was compared with Nomarski images in these cells, most of the FBPase staining was localized to the vacuole at the import time of 20 min (Fig. 7). In this in vitro system, the cells had been shifted to glucose for 20 min at 30°C and then permeabilized for the import reactions. The sequenoster FBPase was detected in the vacuole after an import time of 20 min at 30°C. If we add the glucose shift time of 20 min with the import time of 20 min, a total of 40 min is required to detect FBPase in the vacuole at 30°C. This time course is in close agreement with our in vivo studies that FBPase is found in the vacuole after cells are shifted to glucose for 45 min at 30°C (4, 7). In this reconstituted system, the fluorescence signal is significantly reduced at the import time of 40 min compared with that seen at t = 20 min. This suggests that FBPase is targeted to the vacuole and then degraded by vacuolar proteinases in the reconstituted system.

Recent evidence suggests that FBPase is targeted to intermediate vesicles before uptake by the vacuole (5). In this in vitro system, FBPase staining was in the cytoplasm and also in the vacuole at earlier time points. It is possible that FBPase staining is in the intermediate vesicles at earlier time points. Since we have experienced a high background staining of FBPase in certain yeast strains, we cannot rule out the possibility that FBPase staining in the cytoplasm is nonselective. Given that we only recovered less than 10% of intact vacuole using cell fractionation techniques, a better separation scheme will be necessary to resolve this issue.

The yeast vacuole is the final destination for sorting of vacuolar resident proteins, endocytosis of plasma membrane proteins, and degradation of cytosolic proteins such as FBPase. The in vitro reconstituted system has been used to study homotypic fusion between vacuoles (37), sorting of CPY from the late Golgi to the vacuole (33), and targeting of aminopeptidase I from the cytosol to the vacuole in S. cerevisiae (38). In vitro targeting and processing of aminopeptidase I presents a post-membrane binding transport event, because the precursor aminopeptidase I is labeled in vivo and is associated with some membranous structures before cells are permeabilized. Furthermore, targeting of the precursor aminopeptidase I to the vacuole does not require cytosol (39). In our in vitro reactions, purified FBPase is introduced to the semi-intact cells. FBPase is imported into the vacuole in a process that is stimulated by cytosol. The development of this in vitro FBPase import system provides a functional assay to understand the molecular mechanisms for targeting FBPase from the cytosol to vacuole for degradation.
In Vitro Reconstitution of Glucose-induced Targeting of Fructose-1,6-bisphosphatase into the Vacuole in Semi-intact Yeast Cells
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J. Biol. Chem. 1998, 273:3381-3387.
doi: 10.1074/jbc.273.6.3381

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