Cyclophilin A Mediates Vid22p Function in the Import of Fructose-1,6-bisphosphatase into Vid Vesicles*

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Fructose-1,6-bisphosphatase (FBPase) is synthesized in yeast during glucose starvation but is rapidly degraded in the vacuole following the addition of glucose. FBPase trafficking to the vacuole involves two distinct steps, import into intermediate transport vesicles (Vid vesicles) and Vid vesicle trafficking to the vacuole. FBPase import into Vid vesicles requires the VID22 gene. However, VID22 affects FBPase import indirectly through a cytosolic factor. To identify the required cytosolic component, wild type cytosol was fractionated and screened for proteins that complement Δvid22 mutants cytosol using an in vitro assay that reproduces FBPase import into Vid vesicles. Cyclophilin A (Cpr1p) was identified as a cytosolic protein that mediates Vid22p function in FBPase import. Mutants lacking Cpr1p were defective in FBPase import. Furthermore, the addition of purified Cpr1p restored FBPase import in both the Δcpr1 and the Δvid22 mutants. The cyclophilin A binding pocket is important for Cpr1p function, since cyclophilin A binding-deficient mutants failed to complement FBPase import in Δcpr1 and Δvid22 mutants. The levels of Cpr1p were reduced in the Δvid22 mutants, implying that the expression of Cpr1p is regulated by Vid22p. Our results suggest that Cpr1p mediates Vid22p function and is directly involved in the import of FBPase into Vid vesicles.

The vacuole of the yeast Saccharomyces cerevisiae is homologous to the lysosome of higher eukaryotes and is essential for several cellular processes including pH maintenance, osmoregulation, and protein degradation (1–3). The transport of proteins to the vacuole can occur via several protein trafficking pathways including endocytosis (4), autophagy, cyttoplasm to vacuole transport (5–7), and secretory transport from the late Golgi (2, 3, 8, 9). The most studied example of vacuolar trafficking involving the sorting of the vacuolar luminal protein carboxypeptidase Y (CPY) from the late secretory pathway. CPY is synthesized and translocated into the endoplasmic reticulum. This protein is then transported to the Golgi, where it is sorted by the CPY receptor. Finally, CPY is targeted through the late endosome/prevacuolar compartment on its way to the vacuole. Due to the complexity of this process, the sorting of CPY to the vacuole requires more than 40 VPS genes (3, 10–12).

Cytosolic proteins and organelles can be targeted to the vacuole by the nonselective macroautophagy pathway when S. cerevisiae are starved of nitrogen. Macroautophagy overlaps with the Cvt pathway (5–7, 13). The Cvt and autophagy pathways also share components with the pexophagy pathway (14, 15). Most genes involved in the autophagy or Cvt pathways have been cloned, and their functions have been elucidated. However, the vacuole import and degradation pathway have been identified. For example, a novel ubiquitin-like conjugating system is required for autophagy and Cvt targeting to the vacuole. The Cpr1p is covalently conjugated to phosphatidylethanolamine. This modification affects the membrane distribution of Phag8p and is necessary for the regulation of membrane dynamics in autophagy (17).

A distinct cytosol to vacuole targeting pathway has been studied in our laboratory. Fructose-1,6-bisphosphatase (FBPase) is a key regulatory enzyme in gluconeogenesis in S. cerevisiae. FBPase is induced when yeast cells are grown in medium containing poor carbon sources. However, when glucose-starved cells are shifted to fresh glucose, FBPase is rapidly inactivated and then degraded (18–20). Although the site of FBPase degradation has been a matter of debate (21, 22), a PEPl-dependent degradation of FBPase has been shown by our laboratory and confirmed by an independent research group (23). Using immunofluorescence and immunoelectron microscopy, we showed that FBPase is localized to the vacuole in the pep4 strain (18, 19). In addition, FBPase trafficking to the vacuole has been reconstituted using a semi-intact pep4 strain (20).

Prior to uptake by the vacuole, FBPase is first imported into a novel type of vesicle (24). These Vid vesicles can be purified to near homogeneity and are distinct from ER, Golgi, peroxisomes, mitochondria, COPI, or COPII vesicles (25). Following the import of FBPase into Vid vesicles, these loaded Vid vesicles traffic to the vacuole. There, they fuse with the vacuole and deliver FBPase for degradation. Interestingly, we have identified a role for ubiquitination in the formation of Vid vesicles. The Δubcl ubiquitin mutant exhibits a decrease in the amount of Vid vesicles and a concurrent defect in FBPase import and degradation (26). The levels of Vid vesicles were also reduced when cells overexpressed the R48R63 ubiquitin mutant, a mutation that inhibits the formation of multiubiquitin chains (26). Therefore, it appears that ubiquitin chain formation and ubiquitin conjugation play important roles in the formation of Vid vesicles.

In addition to Ubc1p, several molecules involved in the FBPase degradation pathway have been identified. For example,
A Role for Cpr1p in FBPase Import into Vid Vesicles

In this paper, we fractionated wild type cytosol in order to identify cytosolic factor(s) that can complement Δvid22 mutant cytosol and stimulate FBPase import. We identified cyclophilin A (Cpr1p or Cph1p) as a required factor that mediates the function of Vid22p. Cells lacking Cpr1p exhibited rescued FBPase import into Vid vesicles in vitro and in vivo. However, the FBPase import defect was rescued when purified recombinant Cpr1p was added to in vitro reaction materials derived from either the Δcpr1 or Δvid22 mutants. Cyclosporin A (CsA) binding-deficient mutants failed to complement FBPase import in the Δcpr1 and Δvid22 mutants, indicating that the CsA binding pocket is required for Cpr1p function. Cpr1p levels were significantly reduced in the Δvid22 mutants, suggesting that Vid22p controls the expression of Cpr1p. We propose that Cpr1p mediates Vid22p function and plays a direct role in the import of FBPase into Vid vesicles.

EXPERIMENTAL PROCEDURES

Strains and Reagents—The yeast strains used in this study are listed in Table I. The yeast plasmid pTB3 containing the wild type CPR1 gene on a 2μ vector and bacterial plasmids (Hisα-tagged wild type, H90Y, and G102A cyclophilin A mutants) were obtained from Dr. J. Heitman (Duke University). Rabbit polyclonal antibodies directed against FBPase were purified (Duke University). Rabbit polyclonal antibodies directed against FB-G102A cyclophilin A mutants) were obtained from Dr. J. Heitman.

TABLE I

| Strain       | Genotype                          | Source                        |
|--------------|-----------------------------------|-------------------------------|
| HLY193       | mata leu2–3, 112 his3 trp1 ura3–52  | Euroscarf, Germany            |
| HLY203       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY362       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY683       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY685       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| Y13513       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| Y03513       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY846       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY810       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY884       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |
| HLY879       | mata his3–200 ura3–52 leu2–3, 112 trp1– |                               |

Purification of Cpr1p from Wild Type Cytosol—Wild type cells were grown for 2 days in YPKG (10 liters) and shifted to YPD for 20 min. Cells were harvested, resuspended in 500 ml of buffer (50 mM HEPES, pH 7.2, 5 mM MgSO4, 40 mM (NH4)2SO4, 0.1 mM EDTA) and homogenized with a bead beater at 4°C. All subsequent fractionation steps were carried out at 4°C. Lysates were centrifuged at 13,000 × g, and the supernatant was centrifuged at 200,000 × g for 2 h. The 200,000 × g supernatant was fractionated by ammonium sulfate precipitation at concentrations of 15, 30, and 45%. Samples were centrifuged at 15,000 rpm for 20 min using a S34 rotor, and the precipitates were resuspended in 20 ml of KP buffer (10 mM K2HPO4, pH 7.0, 50 mM NaCl, and 0.02% NaN3). Samples were then dialyzed against the same buffer for 16 h. Aliquots of each fraction (10 μl) were solubilized in SDS sample buffer, resolved by SDS-PAGE, and stained with Coomassie Blue. Aliquots from each fraction (10 μl) were added to the Δvid22 in vitro reaction and tested for their ability to stimulate FBPase import.

Preparative Superose 6 agarose (175 ml in 20% ethanol) was purchased from Sigma. A Superose 6 column was poured and equilibrated with KP buffer. The column was calibrated using thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) as markers. The 30% ammonium sulfate fraction (20 ml) was chosen for Superose 6 fractionation, since this fraction contained the highest complementing activity. A total of 100 fractions (3–4 ml/fraction) were collected from the Superose 6 column. The protein profile (10 μl) was examined by SDS-PAGE and Coomassie Blue staining. Small aliquots (10 μl) from each fraction were tested for their ability to stimulate FBPase import in the in vitro assay.

Fractions 55–75 from the Superose 6 column (80 ml) were pooled and concentrated to 10 ml using a Centricon-10 filter concentrator (Amicon, Beverly, MA). A G75 column (Sigma) was poured and equilibrated with KP buffer. The column was calibrated using 67-kDa bovine serum albumin, 28-kDa carbonic anhydrase, and 14-kDa RNase A, and 12.6-kDa cytochrome c as markers. Following the addition of concentrated protein samples, a total of 60 fractions (3–4 ml/fraction) were collected from the G75 column. Aliquots of the G75 fractions (10 μl) were examined by SDS-PAGE and Coomassie Blue staining. Small aliquots (10 μl) from each of the G75 fractions were added to the Δvid22 in vitro reactions and tested for their ability to stimulate FBPase import.

Fractions 39–45 (25 ml) from the G75 column were pooled and concentrated to 3 ml with a Centricon 10 concentrator. DEAE-agarose was pre-equilibrated with KP buffer, and 200 μl of this slurry was added to the concentrated samples for 2 h. The beads were pelleted by centrifugation at 13,000 × g for 5 min. DEAE beads were washed with 1.5 ml of KP buffer three times, and bound proteins were eluted with 200 μl of 1 M NaCl. The total, flow-through, or eluate fractions (10 μl each) from the DEAE column were examined for protein content by SDS-PAGE and Coomassie Blue staining. These fractions (10 μl) were also tested for FBPase import activity using the in vitro assay.

Purification of GST-Cpr1p—The CPR1 gene was amplified by PCR using the 5’ primer TGGCACTAGTGGTTTCCAAAGTCGTTAGT and the 3’ primer TGGCAATACCTACGGGATGTGCCAAC and cloned into the pYES2.1 TOPO plasmid (Invitrogen, Carlsbad, CA). The CPR1 gene was excised from the plasmid by SalI digestion and ligated into the Xhol site of the pGEX–RI plasmid (ATCC, Manassas, VA). An overnight culture of bacteria (20 ml) expressing GST-Cpr1p was diluted to an OD of 0.3 in 200 ml of LB containing ampicillin (50 μg/ml). Cultures were regrown for 3 h to an OD of 0.8, and the GST-Cpr1p fusion protein was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 3.5 h. Cells were harvested and resuspended in 10 ml of phosphate-buffered saline buffer containing 2 mM EDTA, 0.1% β-mer-

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captoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. Cells were sonicated four times at the maximal speed for 30 s on ice. Lysates were solubilized with 2% Triton X-100 for 15 min and spun at 10,000 × g for 10 min. The supernatant was incubated for 1 h at 4 °C with 300 μl of GST beads (Amersham Biosciences) that had been pre-equilibrated in phosphate-buffered saline. Samples were centrifuged at 1,000 × g for 5 min and washed three times with ice-cold phosphate-buffered saline and one time with thrombin buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% β-mercaptoethanol. Samples were centrifuged at 1,000 × g, and GST beads were resuspended in 2 ml of thrombin buffer containing 6 μg of thrombin. Samples were incubated for 40 min at 22 °C and then centrifuged. Total lysates and the final supernatants were collected and proteins were examined by SDS-PAGE and Coomassie Blue staining.

**Purification of His₆-tagged Vid22p, Cpr1p, H90Y, and G102A Proteins**—Plasmids expressing His₆-tagged versions of wild type Cpr1p, H90Y, and G102A mutant proteins were utilized. These proteins were expressed and purified from bacterial strains as described (28). A Vid22p-His₆ fusion construct was cloned into pYES21 TOP1 plasmids and transformed into the Δvid22 yeast strain. Yeast lysates (50 ml) containing recombinant proteins were solubilized in 500 μl of Ni⁺⁺ binding buffer containing 2% Triton X-100 and purified using the protocol provided by Novagen. Collected fractions (total, unbound, and bound) were examined for protein content via SDS-PAGE and Western blotting. The Bio-Rad protein assay was used to determine protein concentrations.

**Miscellaneous Assays**—FBPase distribution in the high speed supernatant and high speed pellet fractions was determined as described (26). FBPase activity was measured as described (18).

**RESULTS**

**Vid22p Regulates FBPase Import through the Cytosolic Fraction**—The Δvid22 yeast strain displays a defect in the degradation of FBPase following a glucose shift. FBPase accumulates in the cytosol of the Δvid22 strain, suggesting that FBPase targeting to Vid vesicles requires the VID22 gene.

Since Vid22p is a plasma membrane protein, it is not known how it regulates FBPase import into Vid vesicles. To address this question, we performed experiments utilizing our in vitro assay that recapitulates the import of FBPase into Vid vesicles (27). In this assay, cytosol and Vid vesicle fractions are isolated from strains that have been shifted to glucose for 20 min. The endogenous FBP1 gene was deleted from these strains so that a known quantity of purified FBPase could be added to the in vitro reaction mixtures, which also contained ATP and an ATP regenerating system. Following proteinase K treatment, FBPA import was defined as the fraction of FBPase that was regenerating system. Following proteinase K treatment, FBPase activity was measured as described (18).

As is shown in Fig. 1A, the combination of wild type cytosol and Vid vesicles resulted in the import of FBPase into Vid vesicles (lane 1). In contrast, the combination of Δvid22 cytosol and Vid vesicles was not competent for FBPase import (lane 2). To identify the defective component in the Δvid22 strain, we utilized various combinations of cytosol and Vid vesicles isolated from the wild type and Δvid22 strains. The combination of wild type cytosol and Δvid22 Vid vesicles was competent for FBPase import (lane 3). Therefore, it appears that Vid vesicles from the Δvid22 strain are functional. In contrast, the combination of Δvid22 cytosol and wild type Vid vesicles did not simulate FBPase import (lane 4). This suggests that the Δvid22 strain has defective cytosol that cannot drive the import of FBPase into Vid vesicles.

**Vid22p Stimulates FBPase Import Indirectly**—Since Vid22p is a plasma membrane protein, it may exert its effect on FBPase import in an indirect manner. To test this, the VID22 gene was fused with a His₆-coding DNA under a GAL1-inducible promoter and transformed into the Δvid22 strain. The fusion protein retained its function, since the transformed cells exhibited a wild type FBPase degradation phenotype when the expression was induced. In contrast, in the absence of induction, the cells did not degrade FBPase (not shown). To determine whether purified Vid22p is capable of stimulating FBPase import, His₆-tagged Vid22p was purified on a nickel column under native conditions, using detergent-solubilized lysates from transformed yeast strains. As is shown in Fig. 1B, most of the Vid22p was in the eluate fraction (lane 2), and a minimal amount was in the flow-through fraction (lane 3). As expected, most of the control protein Pma1p was in the flow-through (lane 3), but very little was found in the eluate fraction (lane 2). The total, elute, and flow-through fractions were added to the in vitro reaction mixtures consisting of cytosol and Vid vesicles derived from the Δvid22 mutant. When FBPase import was examined, the stimulatory activity was in the flow-through fraction, although this fraction contained very low levels of Vid22p (Fig. 1B). In contrast, the high levels of Vid22p in the eluate fraction did not stimulate FBPase import. Therefore, Vid22p appears to regulate FBPase import indirectly.

**Wild Type Cytosol Complements Δvid22 Mutant Cytosol in FBPase Import**—The defect in the Δvid22 cytosolic fraction most likely represents the lack of necessary constituents in this strain. To test this idea, we again performed the in vitro assay utilizing the Δvid22 cytosol and Vid vesicles. In these experiments, however, wild type cytosol was also included in the reaction mixture. As is shown in Fig. 1C, the addition of wild type cytosol to Δvid22 cytosol resulted in the import of FBPase (lane 3). Thus, this suggests that there is a cytosolic component present in wild type cytosol that can complement Δvid22 cytosol. Along these lines, we have identified Ssa2p as an essential cytosolic protein that is required for the import of FBPase into Vid vesicles (27). To determine whether Ssa2p is the missing cytosolic protein in the Δvid22 mutants, we combined cytosol obtained from a Δssa2 mutant strain with Δvid22 mutant cytosol in the in vitro reaction. If Ssa2p is the missing cytosolic component in Δvid22 cells, then the addition of cytosol from the Δssa2 strain should not stimulate import. However, a high level of FBPase import was observed under these experimental conditions, suggesting that the Δssa2 mutant strain contains essential cytosolic proteins that complement Δvid22 mutant cytosol (lane 4). Thus, this result suggests that Ssa2p is not the missing cytosolic factor in the Δvid22 mutant.

**Purification of Cpr1p from Wild Type Cytosol**—Since wild type cytosol contains the essential component that is absent from the Δvid22 strain, we began a series of fractionation steps in order to isolate and identify this component (Fig. 2). Wild type cells were shifted to glucose for 20 min and harvested. Cell lysates were subjected to differential centrifugation, and the final 200,000 × g supernatant (cytosol) was fractionated via precipitation with increasing concentrations of ammonium sulfate. Precipitants were dialyzed, and small aliquots were then tested in our in vitro assay utilizing Δvid22 components. The 30% ammonium sulfate cytosol was the most effective in restoring FBPase import, whereas the 15 or 45% precipitates had little stimulatory effect (Fig. 2A).

The 30% ammonium sulfate precipitant was subjected to further fractionation using Superose 6 sizing chromatography (Fig. 2B). Fractions were collected from the Superose 6 column and tested in the in vitro assay to identify those that could complement FBPase import in the Δvid22 strain. In order to estimate the size of the stimulatory proteins, the Superose 6 column was calibrated with molecular weight markers ranging from 29 to 660 kDa. The complementing activity was found in...
the last few fractions that eluted after the 29-kDa marker, suggesting that the stimulatory proteins were smaller than 29 kDa. To further purify the complementing activity, the stimulatory fractions from the Superose 6 column were pooled, concentrated by Centricon 10 filtration, and subjected to further fractionation on a G75 sizing column (Fig. 2C). Fractions collected from the G75 column were tested for their ability to stimulate import in the H9004vid22 mutants. The highest activity was found in fractions 39–45. These stimulatory fractions were pooled, concentrated, and subjected to DEAE ionic exchange chromatography (Fig. 2D). The total, flow-through, and DEAE-bound fractions were examined by SDS-PAGE and Coomassie Blue staining. Aliquots of these fractions were also tested for their effects on FBPase import using the in vitro assay. The DEAE eluate fraction was the most effective in complementing FBPase import in the Δavid22 mutants. In contrast, the flow-through fraction had little stimulatory effect on FBPase import. When the eluate fraction was examined by Coomassie Blue staining, the most prominent band was ~17 kDa. To identify this protein, the band was excised from the gel and subjected to in-gel trypsin digestion followed by matrix-assisted laser desorption/ionization analysis. The protein was subsequently identified as cyclophilin A (Cpr1p or Cph1p), a protein that exhibits peptidyl prolyl cis-trans isomerase activity (29). Cpr1p is a member of the immunophilin family and is the major cyclosporin A receptor protein (29–31).

Cpr1p Is Required for FBPase Import into Vid Vesicles—To determine whether Cpr1p plays a role in the import of FBPase into Vid vesicles, we utilized a yeast strain in which the endogenous CPR1 gene had been deleted. A culture of the H9004cpr1 strain was glucose-starved and shifted to glucose for 0 or 60 min. Cells were harvested, and lysates were subjected to differential centrifugation. The distribution of FBPase in the high speed supernatant (enriched for cytosol) and high speed pellet (enriched for Vid vesicles) fractions was then determined. As a control, we utilized the H9004vid24 mutant that has normal FBPase import but blocks targeting of FBPase from the Vid vesicles to the vacuole. In the H9004vid24 mutant, FBPase was found in the high speed supernatant (S) fraction at t = 0 min (Fig. 3A). However, after a shift of this strain to glucose for 60 min, a significant amount of FBPase was detected in the Vid vesicle-containing high speed pellet (P) fraction, suggesting that a portion of FBPase is targeted to Vid vesicles in this mutant. In the Δcpr1 mutant, FBPase was also found in the
high speed supernatant fraction during glucose starvation. However, FBPase remained in the supernatant fraction after the Δcpr1 strain was shifted to glucose for 60 min (Fig. 3A).

Therefore, the absence of the CPR1 gene resulted in accumulation of FBPase in the cytosol and blocked the import of FBPase into Vid vesicles.

If CPR1 is required for FBPase targeting to Vid vesicles in vivo, then FBPase import in vitro might also be affected in the Δcpr1 mutant. To determine whether this is the case, we produced cytosol and Vid vesicles from the Δcpr1 strain and examined them for their ability to stimulate FBPase import. The combination of Δcpr1 cytosol and Δcpr1 Vid vesicles did not support import of FBPase (Fig. 3B, lane 2). To determine the site of this defect, we examined various combinations of cytosol and Vid vesicles derived from wild type and Δcpr1 strains. The combination of wild type cytosol and Δcpr1 Vid vesicles was
concentrations of bovine serum albumin to the pendent manner (Fig. 4). The import of FBPase was stimulated in a dose-dependent manner by the absence of Cpr1p. Therefore, the defects observed for Cpr1p mutants in FBPase import are not due to an increased level of protein—specifically, the addition of a purified Cpr1p fraction did not stimulate the import of FBPase into Vid vesicles (Fig. 4). We can next express GST-Cpr1p in E. coli (Fig. 4B, lane 1) and purified the fusion protein using a glutathione column. Following cleavage of the fusion protein with thrombin, Cpr1p was collected in the supernatant. As shown by Coomassie Blue staining, two prominent bands were observed (Fig. 5), which were recognized by anti-Cpr1p antibodies (not shown). As is shown in Fig. 5C, when wild type His$_6$-tagged Cpr1p was added to the Vidvesicles reaction mixture, the import of FBPase was stimulated in a dose-dependent manner by the absence of Cpr1p. Therefore, the effects observed following Cpr1p addition are specific and are not due to the presence of increased protein concentrations.

The CsA Binding Pocket Is Important for Cpr1p Function—Members of the cyclophilin family were discovered based on their ability to bind to the immunosuppressant drug CsA. Furthermore, the binding of CsA to cyclophilins often results in their inactivation (29, 31—34). Therefore, if functional Cpr1p is required for FBPase import, the addition of CsA might inhibit FBPase import in the wild type in vitro reaction. When various concentrations of CsA were added to our wild type in vitro reaction components, the import of FBPase into Vid vesicles was inhibited (Fig. 5A). This result supports the idea that Cpr1p is involved in the import of FBPase into Vid vesicles.

To determine whether CsA binding is important for the function of Cpr1p in FBPase import, we attempted to utilize yeast cpr1 mutants that perturb the CsA binding pocket. However, these mutant strains exhibit growth defects and result in petite cells (36). Furthermore, the expression of the mutant proteins varied. For example, the H90Y mutant protein was produced at ∼25% of the wild type level (29). Therefore, we could not distinguish whether a reduced Cpr1p level or a mutation in the CsA binding pocket was responsible for the FBPase import defect seen for the H90Y mutant protein.

As an alternative approach, we expressed various mutant forms of Cpr1p in bacteria and purified these proteins that have perturbations in the CsA binding pocket. Equal amounts of these proteins were added to the in vitro reaction in order to examine their effects on in vitro FBPase import. For these experiments, bacteria were transformed with plasmids expressing the His$_6$-tagged wild type, H90Y, and G102A proteins. These proteins were induced and purified on a nickel column. When the proteins were examined by SDS-PAGE and Coomassie Blue staining, two prominent bands were observed (Fig. 5B). Note that the fast migrating bands may result from the degradation of a portion of the proteins during the purification procedure, since both bands were recognized by anti-Cpr1p antibodies (not shown). As is shown in Fig. 5C, when wild type His$_6$-tagged Cpr1p was added to in vitro material derived from the cpr1 mutants, FBPase import was stimulated. In contrast, neither purified H90Y protein nor G102A protein supported FBPase import under the same conditions. These studies suggest that the CsA binding pocket is required for Cpr1p function in FBPase import. On the other hand, prolyl isomerase activity may not play an important role in this process. Purified G102A protein has been shown to have prolyl isomerase activity in vitro comparable with that of wild type Cpr1p. By contrast, purified H90Y exhibits significantly reduced prolyl isomerase activity in vitro (29). However, neither of these mutant proteins could stimulate FBPase import in vitro. Thus,
these results suggest that prolyl isomerase activity of Cpr1p is not sufficient to support FBPase import.

The Levels of Cpr1p are Reduced in the ∆cpr1 Strain—The DEAE-bound material from our fractionation experiments was highly enriched in Cpr1p (see Fig. 2). Thus, the ability of this material to complement FBPase import in the ∆cpr1 in vitro reaction mixture suggests that Cpr1p is either absent or is expressed at low levels in this strain. To test this idea, the expression of Cpr1p was compared for wild type and ∆cpr1 strains. Cells were glucose-shifted and harvested at various time points, and cell lysates were examined for the expression of Cpr1p by immunoblotting with anti-Cpr1p antibodies. As is shown in Fig. 6, wild type strains contained high levels of Cpr1p, while there was no detectable Cpr1p in the ∆cpr1 lysates. Interestingly, the ∆vid22 strain contained less than 10% of the Cpr1p protein observed for wild type cells when equivalent amounts of cellular protein were used. Note that the levels of another cytosolic protein, glucose-6-phosphate dehydrogenase, were similar in the ∆cpr1, ∆vid22, and wild type strains (Fig. 6). Therefore, Cpr1p levels are selectively reduced in the ∆vid22 mutant strain.

Cpr1p Rescues the Defect of the ∆vid22 Strain—If a decrease in Cpr1p levels is responsible for a reduced FBPase import in the ∆vid22 strain, then the addition of exogenous Cpr1p should also rescue the FBPase import defect in this strain. To test this, Cpr1p was purified (see Fig. 4) and added to our in vitro reaction materials derived from the ∆cpr1 mutants. As is shown in Fig. 7A, increasing amounts of Cpr1p complemented ∆cpr1 mutant components in FBPase import. In contrast, the addition of a control protein (bovine serum albumin) did not restore FBPase import in the ∆cpr1 mutant. Therefore, the import of FBPase in the ∆vid22 mutants can be rescued by the addition of exogenous Cpr1p in vitro.

Next, we examined whether CsA binding or the prolyl isomerization activity of Cpr1p plays a role in FBPase import in the ∆vid22 strain. His6-tagged wild type Cpr1p, H90Y, and

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**Fig. 4. Purified recombinant Cpr1p restores FBPase import into the Δcpr1 mutant strain.** A, total (T), flow-through (FT), and eluate (E) fractions from the final DEAE purification step were obtained as described in the legend to Fig. 2. These fractions were added to the in vitro reactions containing cytosol and vesicles derived from Δcpr1 mutants to examine them for FBPase import. B, a GST-Cpr1p fusion construct was induced in bacteria, and total lysates were examined via SDS-PAGE and Coomassie Blue staining (lane 1). GST-Cpr1p was purified on a glutathione column followed by cleavage of the fusion protein with thrombin to release Cpr1p into the supernatant (lane 2). C, purified Cpr1p was added to the reaction mixture at increasing concentrations to determine their effects on FBPase import in Δcpr1 mutants. Bovine serum albumin was added to the Δcpr1 mutant mixture as a control.

**Fig. 5. The CsA binding pocket is required for Cpr1p function.** A, cyclosporin A inhibits FBPase import. Increasing concentrations of cyclosporin A were added to the in vitro reaction material derived from a wild type yeast strain. FBPase import was measured as described under “Experimental Procedures.” B, His6-tagged wild type Cpr1p (lane 1), H90Y mutant (lane 2), and G102A (lane 3) mutant proteins were purified from bacterial lysates using a nickel column and examined for protein concentrations. The same amounts of proteins (2 μg) were resolved by 15% polyacrylamide gels and stained with Coomassie Blue. C, purified wild type Cpr1p, H90Y, and G102A proteins were added to in vitro reactions containing cytosol and vesicles derived from the Δcpr1 mutants to examine them for FBPase import.
G102A proteins were purified as described in the legend to Fig. 5. These proteins were added to the in vitro reaction components derived from the Δavid22 strains. The addition of wild type Cpr1p complemented Δavid22 mutants in FBPase import (Fig. 7B), in agreement with our previous results. However, the addition of the H90Y or the G102A mutant proteins did not (Fig. 7B). Therefore, the CsA binding pocket is necessary for the function of Cpr1p in complementing FBPase import in the Δavid22 mutant.

**FBPase Is Inactivated in Response to Glucose in the Δcpr1 Mutant**—FBPase is inactivated rapidly after cells are shifted to glucose. This event occurs prior to FBPase import into Vid vesicles and may be a necessary step for the import process. Although the mechanism has not been completely established, inactivation appears to be mediated by a c-AMP dependent signal transduction pathway. Interestingly, both phosphorylation and ubiquitination of FBPase have been reported following the addition of glucose (22, 23). At present, however, it is unknown whether Cpr1p plays a role in FBPase import through its modulation of FBPase activity. To test this, FBPase activity was compared between wild type and the Δcpr1 mutant strain. As is shown in Fig. 8, FBPase activity was similar for wild type and Δcpr1 mutant cells when maintained under glucose starvation conditions. Likewise, FBPase activity de-
FBPase activity is unlikely to be regulated by Cpr1p. Therefore, we did not detect a significant change in FBPase activity under these experimental conditions (data not shown). As an additional test, we incubated purified FBPase with purified Cpr1p either in the absence or presence of cytosol. Again, we did not detect a significant change in FBPase activity under these experimental conditions (data not shown). Therefore, FBPase activity is unlikely to be regulated by Cpr1p.

**DISCUSSION**

During the degradation process, FBPase is targeted from the cytosol to Vid vesicles before it is subsequently delivered to the vacuole. Therefore, there are at least two groups of proteins that are involved in FBPase trafficking. The first group plays a role in the sequestration of FBPase into Vid vesicles, while the second group is involved in FBPase trafficking from Vid vesicles to the vacuole. The FBPase import step requires the assistance of at least two cytosolic proteins. We have shown previously that the heat shock protein Ssa2p is necessary for the import of FBPase into Vid vesicles. Cyclophilins are known to catalyze cis-trans peptidyl prolyl isomerization, at least under in vitro conditions (29, 30, 32–35). These proteins are highly conserved from bacteria to humans and are found in multiple compartments. In yeast, eight cyclophilin members have been identified. Deletion of one or all eight of these genes still results in viable cells, suggesting that these genes are nonessential for growth (37). Some of these cyclophilins are associated with organelles. For instance, Cpr2p, Cpr4p, and Cpr5p reside in the ER and may be involved in some aspects of ER functions (37). Another cyclophilin homologue, Cpr3p, is localized in the mitochondrial matrix and is known to be involved in the folding of proteins in the mitochondrial matrix (33). However, crp3 null mutants exhibit a normal rate of protein import into mitochondria, suggesting that Cpr3p does not play a role in the import process (33). In this paper, we provide evidence showing that Cpr1p is involved in the import of FBPase into Vid vesicles. Furthermore, Cpr1p mediates the function of Vid22p in this import process. The deletion of the CPR1 gene resulted in a defect in FBPase import in vitro and in vivo. Moreover, the addition of purified Cpr1p complemented Δcrp1 and Δvid22 mutants in FBPase import. Taken together, these results suggest that Cpr1p plays a direct role in the import process.

Cpr1p catalyzes prolyl isomerization in vitro (29). However, prolyl isomerization may not be required for Cpr1p function in FBPase import. The G102A mutant retains prolyl isomerase activity comparable with wild type Cpr1p (28). However, this mutant protein failed to stimulate FBPase import, suggesting that prolyl isomerase activity is not sufficient for Cpr1p function, at least in the FBPase import process. A similar conclusion has been reported previously with mitochondrial Cpr3p mutants that have reduced prolyl isomerase activity. The R73A and the H144Q mutants have defective prolyl isomerization activity in vitro. However, they retain biological functions in vivo and catalyze protein refolding effectively in vitro (30). Therefore, prolyl isomerase activity of cyclophilins may be dispensable for their physiological functions.

Cyclophilins may exert this function by forming stoichiometric protein-protein complexes rather than catalytic enzyme-substrate interactions. In *Drosophila*, the cyclophilin homologue, NinaA, is required for proper maturation of the major rhodopsin Rh1 (38–40). NinaA forms a stable complex with rhodopsin and co-localizes with Rh1 in the secretory vesicles, suggesting that Rh1 may require ninaA as it travels through the distal compartments of the secretory pathway (38). Furthermore, ninaA mutations inhibit Rh1 transport from the ER, leading to accumulation of ER cisternae (40). Another example of protein-protein interaction comes from studies showing that human cyclophilin A and the human immunodeficiency virus 1 GAG protein form a complex (32, 41, 42). CsA disrupts the cyclophilin A-GAG interaction, leading to the production of a human immunodeficiency virus 1 virus lacking cyclophilin A. The resulting virions have defects at an early stage after viral entry into the infected cells (41, 42). A protein-protein interaction has also been demonstrated whereby Cpr6p and Cpr7p interact directly with Hsp90 through the tetratricopeptide-containing C terminus (43). Cpr6p and Cpr7p are part of the Hsp90 complex that is involved in the steroid hormone signal transduction pathway (43).

Cpr1p has been shown to interact with Sap30 and Rpd3p in vitro (44). This interaction is important for the regulation of gene silencing by the Sin3-Rpd3 histone deacetylase (31, 44). Since the majority of Cpr1p is in the cytosol, the cytosolic pool of Cpr1p may have roles that are distinct from the regulation of gene silencing that occurs in the nucleus. Our in vitro assay indicates that the cytosolic portion of Cpr1p is involved in the FBPase import step. When the CPR1 gene is deleted in cells, FBPase import is impaired. In the Δcrp1 mutant, cytosol is the defective compartment responsible for the reduced import. Moreover, the addition of purified Cpr1p complemented the Δcrp1 mutant cytosol in FBPase import. Therefore, the cytosolic fraction of Cpr1p plays a direct role in the initial step of FBPase protein trafficking, a process that occurs in the cytoplasm.

We attempted to examine whether Cpr1p interacts directly with FBPase, using various approaches. Unfortunately, no specific interaction of Cpr1p with FBPase was detected using co-immunoprecipitation experiments or cross-linking with various cross-linkers. Likewise, we could not detect specific binding of FBPase to purified GST-Cpr1p immobilized on glutathione beads or to purified His6-Cpr1p immobilized on nickel beads (not shown). In addition, Cpr1p does not appear to regulate FBPase activity, since FBPase was inactivated normally in response to glucose in the absence of the CPR1 gene. Therefore, Cpr1p is unlikely to regulate FBPase import through a
direct protein-protein interaction with FBPase or through the modulation of FBPase activity. However, there is a formal possibility that Cpr1p interacts with other unidentified proteins in order to stimulate FBPase import. This possibility remains to be tested in the future.

When the CsA binding pocket was inactivated, the mutated proteins failed to complement Δcpr1 and Δvid22 mutants in FBPase import. This suggests that the CsA binding pocket is important for Cpr1p function in FBPase import. It is not known how CsA interferes with the function of Cpr1p in FBPase import. One possibility is that CsA binding directly inactivates Cpr1p. Since Cpr1p can interact with other proteins, it is also possible that these protein-protein interactions are inhibited by CsA binding. Conversely, the binding of CsA to Cpr1p may lead to the association of Cpr1p with other proteins. This type of interaction might reduce the amount of active Cpr1p that could participate directly in FBPase import, thereby inhibiting the FBPase import process.

In addition to the allosteric regulation of Cpr1p by CsA, Cpr1p can also be regulated at the level of protein expression. We showed that Vid22p controls Cpr1p expression, since Cpr1p levels were reduced significantly in the absence of Vid22p. Since Vid22p is a plasma membrane protein, one possible explanation is that Vid22p is involved in a cascade of signal transduction events that ultimately lead to the control of Cpr1p expression.

Based upon our results from this and previous studies, we propose that the FBPase degradation pathway can be divided into distinct trafficking steps (Fig. 9). Following a glucose shift, Vid vesicles are formed via a process that requires the ubiquitin-conjugating enzyme Ubc1p. In the absence of Ubc1p, the levels of Vid vesicles are reduced, and FBPase import is impaired. FBPase is imported into Vid vesicles in a process that is dependent upon the heat shock protein Ssa2p. The plasma membrane protein Vid22p also plays an important but indirect role in FBPase import. Vid22p regulates the levels of Cpr1p, which in turn stimulates FBPase import into Vid vesicles. Finally, a number of other gene products are likely to play a role in the downstream FBPase trafficking events. At present, only Vid24p has been identified as playing a role in this step of the FBPase degradation pathway. Accordingly, our goal is to identify and characterize more genes that participate in each step of the FBPase degradation pathway.

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