The Type 1 Phosphatase Reg1p-Glc7p Is Required for the Glucose-induced Degradation of Fructose-1,6-bisphosphatase in the Vacuole*

Dong-Ying Cui, C. Randell Brown‡, and Hui-Ling Chiang

From the Department of Cellular and Molecular Physiology, Penn State University College of Medicine, Hershey, Pennsylvania 17033

Protein phosphatases play an important role in vesicular trafficking and membrane fusion processes. The type 1 phosphatase Glc7p and its regulatory subunit Reg1p were identified as required components in the glucose-induced targeting of the key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) to the vacuole for degradation. The interaction of Reg1p with Glc7p was important for the transport of FBPase from intermediate vacuole import and degradation (Vid) vesicles to vacuoles. The gle7-T152K mutant strain exhibited a reduced Reg1p binding along with defects in FBPase degradation and Vid vesicle trafficking to the vacule. In this mutant, Vid vesicles were the most defective components, whereas the vacuole was also defective. Shp1p and Glc8p regulate Glc7p phosphatase activity and are required for FBPase degradation. In the Δshp1 and Δgcl8 strains, Reg1p-Glc7p interaction was not affected, suggesting that phosphatase activity is also necessary for FBPase degradation. Similar to those seen in the gle7-T152K mutant, the Δshp1 and Δgcl8 mutants exhibited severely defective Vid vesicles, but partially defective vacuoles. Taken together, our results suggest that Reg1p-Glc7p interaction and Glc7p phosphatase activity play a required role in the Vid vesicle to vacuole-trafficking step along the FBPase degradation pathway.

Regulation of the protein degradation process plays an essential role in cells. Although each cellular protein has its own intrinsic rate of turnover, this can be altered dramatically depending upon changes in the cellular environment. For example, in Saccharomyces cerevisiae, proteins are targeted to the vacuole (the yeast homologue of the lysosome) following periods of nitrogen starvation by autophagy (1–3). This process appears to be nonspecific, because cytosolic proteins are engulfed non-selectively in autophagosomes and delivered to the vacuole (2–5). Serum starvation also enhances the rate of turnover of cellular proteins in mammalian cells, by targeting proteins for degradation in the lysosome (6, 7). This is a more selective process, in that proteins with a KFERQ motif are delivered to the vacuole in a molecular chaperone-mediated manner (8).

The process of protein degradation also removes misfolded proteins, or proteins that are no longer required for a specific function. Along these lines, certain gluconeogenic enzymes are essential when yeast are grown in poor carbon sources, but they are rapidly inactivated when cells are replenished with fresh glucose (9, 10). This process is critical for the cell, because it helps to prevent energy futile cycles. At present, the mechanisms of inactivation and degradation are not well established for most gluconeogenic enzymes. However, both vacuolar and proteasome-dependent degradation pathways have been described for fructose-1,6-bisphosphatase (FBPase) (11–13). The vacuolar degradation pathway has been shown to require a vesicle-dependent trafficking step (14–16). FBPase is first targeted to intermediate vesicles and then to the vacuole for degradation. These vacuole import and degradation (Vid) vesicles have been purified to near homogeneity and partially characterized (14). The formation of Vid vesicles requires the ubiquitin-conjugating enzyme Ubc1p (17). FBPase import into Vid vesicles is dependent on the heat shock protein Ssa2p (18), cyclophilin A (19), and Vid22p (20). Following FBPase import into Vid vesicles, this protein is then delivered to the vacuole for degradation via a Vid24p-, Ypt7p-, and SNARE-mediated process (21).

Membrane trafficking events require the participation of various kinases and phosphatases (1–3, 22–25). For example, Glc7p is the catalytic subunit of the protein phosphatase type 1 (PP1) and plays a critical role in several cellular trafficking events, including homotypic vacuole fusion, endoplasmic reticulum-to-Golgi transport and endocytic transport (22). Specificity of Glc7p is dictated by regulatory subunits that target the catalytic subunit to various substrates for different functions under different growth conditions. As an example, Gac1p and Pig1p target Glc7p to glycan accumulation (26, 27). Reg2p targets Glc7p to growth and cell cycle progression (28), whereas the formation of the Bni4p-Glc7p complex is required for cytokinesis (29). Bud14p interacts with Glc7p and is involved in cellular morphogenesis during vegetative growth (30). Gip1p targets Glc7p to participate in meiotic and sporulation processes (31). The regulatory subunit Reg1p has been shown to strongly interact with Glc7p in the presence or absence of glucose (36), and this interaction directs Glc7p to a number of cellular processes, including glucose repression, growth, and glycolytic accumulation (28, 32–37). In addition to proteins that regulate Glc7p specificity, Glc7p-PP1 activity can also be modulated by Shp1p and Glc8p (38, 39). SHP1 (suppressor of high copy PP1) was identified as a mutation that suppresses the lethality caused by Glc7p overexpression (38). Mutations in the

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† To whom correspondence should be addressed: Dept. of Cellular and Molecular Physiology, Penn State University College of Medicine, 500 University Dr., Hershey, PA 17033; Tel.: 717-531-0859; Fax: 717-531-7667; E-mail: crb13@psu.edu.

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SHP1 gene lead to a marked reduction of the Glc7p-PP1 activity (38). Likewise, deletion of the GLC8 gene also significantly decreases Glc7p-PP1 activity (39). Under the same conditions, deletion of other regulatory subunits such as Reg1p, Reg2p, and Gac1p had minimal effects on Glc7p-PP1 activity (39). At present, no role has been established for any phosphatase or phosphatase regulators in the trafficking of FBPase to the vacuole. However, Reg1p has been reported to play a role in the proteasome-dependent degradation of FBPase, presumably via its action as a signaling molecule (40).

The interaction of Glc7p with its regulatory subunits has been studied in detail. Analysis of the crystal structure revealed a hydrophobic groove on the surface of PP1c that is important for PP1c interaction with the PP1c-binding motif (R/K)(V/I)XP found in many targeting subunits of PP1c (41). Site-directed mutagenesis studies demonstrated that this hydrophobic groove is indeed necessary for Glc7p binding to (R/K)(V/I)XP-containing regulatory subunits, including those in Reg1p and Gac1p. This hydrophobic groove is not only important for Glc7p binding to the motif, but it is also necessary for Glc7p biological activity and for its proper subcellular localization (42). In addition to the hydrophobic groove, charged residues on the surface of Glc7p are important for its functions. For glucose derepression and cell cycle control, critical residues have been mapped to specific regions of the protein. For glycogen synthesis and sporulation, these charged residues are more widely distributed over the protein surface (43).

We have identified a number of proteins that participate in the degradation of FBPase. These include the molecular chaperone Ssa2p (18), the immunophilin cyclophilin A (19), and a plasma membrane protein Vid22p (20). Likewise, Vid24p (16) and various members of the SNARE and homotypic fusion vacuole protein sorting families of proteins (21) play roles in this process. To identify additional proteins that are involved in the FBPase degradation pathway, we screened a yeast GST library in search of proteins that bind to FBPase. The phosphatase regulatory subunit Reg1p was identified as a putative FBPase-binding protein. FBPase degradation was defective in a Δreg1 strain, suggesting that Reg1p plays some role in the FBPase degradation. An interaction between Reg1p and Glc7p appears necessary for FBPase trafficking. A yeast strain harboring a mutant form of Glc7p that inhibits the Reg1p interaction was defective in both FBPase trafficking and degradation. The Δgcl8 and Δshp1 strains that affect the catalytic activity of Glc7p also exhibited impaired degradation of FBPase. The functions of Vid vesicles and vacuoles were affected by mutations in the GLC7, SHP1, and GLC8 genes. Taken together, our results indicate that Reg1p-Glc7p plays an essential role in the trafficking of Vid vesicles to the vacuole.

EXPERIMENTAL PROCEDURES

Yeast Strains, Primers, and Antibodies—S. cerevisiae strains used in this study are listed in Table I. The deletion strains derived from BY4742 were from Euroscarf (Euroscarf, Germany), and the yeast GST strains in this study were from Dr. Eric Phizicky (University of Rochester, Indiana). Yeast strains used in Table I are listed in Table I. The deletion strains derived from BY4742 were from Euroscarf (Euroscarf, Germany), and the yeast GST strains in this study were from Dr. Eric Phizicky (University of Rochester, Indiana). Yeast strains used in this study are listed in Table I. The deletion strains derived from BY4742 were from Euroscarf (Euroscarf, Germany), and the yeast GST strains in this study were from Dr. Eric Phizicky (University of Rochester, Indiana).
ester School of Medicine) via Dr. A. Hopper (Penn State College of Medicine). The glc7-108, glc7-133, glc7-132, and glc7-127 mutants were gifts from Dr. K. Tatchell (Louisiana State University Health Sciences Center). They are congenic to strain JC482 (MATα ura3–52 leu2 his4). Rabbit polyclonal antibodies directed against FBPase were raised by the Berkeley Antibody Company using purified proteins. Mouse monoclonal anti-HA and anti-myc were purchased from Covance with pulse–chase assay method (SC media without t-methionine, but containing 2% ethanol) and then incubated in the same media for 4–5 h. [35S]Met and [35S]Cys were added to the cells to the final concentration of 45 μCi/ml. After 5–6 h of labeling, the cells were collected by centrifugation in SC media containing 10 μg/ml t-methionine, 10 μg/l t-cysteine, and 2% glucose. At the indicated time points, 1-ml aliquots of cells were collected and fixed by the addition of trichloroacetic acid (5%). After washing the cells three times with cold water, cells were lysed and subjected to immunoprecipitation as described above. The 32P-labeled proteins were quantitated using a PhosphorImager (Amersham Biosciences). 

**Screening for FBPase Interacting Proteins**—Yeast strains containing a GST tag on selected open reading frames, or pools of these strains, were grown for 2 days in YPKG (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 10 g/l potassium acetate, 5 g/l dextrose) media (5 ml) and shifted to YPD (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 20 g/l dextrose) for 30 min as described (16, 21). Cells were lysed in GST binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 supplemented with 1 mM phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation at 10,000 × g at 4 °C. Supernatants were incubated with 2% Triton X-100 for 30 min at room temperature. Insoluble cell debris was removed by centrifugation (13,000 × g for 20 min at 4 °C). Supernatants were then incubated with pre-washed glutathione-Sepharose 4B beads (Amersham Biosciences) for 3 h at 4 °C. The beads were collected by centrifugation at 500 × g for 1 min, and then washed five times with buffer. The washed beads were separated by SDS-PAGE. FBPase-bound to GST fusion proteins were detected by Western blotting procedures using anti-GST antibodies.

**Reg1p Is Required for FBPase Degradation**—We have established a model for the FBPase degradation pathway that consists of at least two steps. The first step involves the targeting of FBPase to Vid vesicles, whereas in the second step, Vid vesicles traffic to the vacuole for degradation (16). In vitro pulse–chase experiments, we utilized a modification of the protocol described by Horak and Wolf (40). Cells were grown in SC media without t-methionine to A600 = 5. Cells then were washed twice with pulse–chase assay media (SC media without t-methionine, but containing 2% ethanol) and then incubated in the same media for 4–5 h. [35S]Met and [35S]Cys were added to the cells to the final concentration of 45 μCi/ml. After 5–6 h of labeling, the cells were collected by centrifugation in SC media containing 10 μg/ml t-methionine, 10 μg/l t-cysteine, and 2% glucose. At the indicated time points, 1-ml aliquots of cells were collected and fixed by the addition of trichloroacetic acid (5%). After washing the cells three times with cold water, cells were lysed and subjected to immunoprecipitation as described above. The 32P-labeled proteins were quantitated using a PhosphorImager (Amersham Biosciences).
ited a defect in FBPase degradation. By contrast, neither Δmet25 nor Δynr065C strains was defective in FBPase degradation. We next performed pulse-chase labeling and immunoprecipitation analysis to confirm that FBPase degradation was impaired in the Δreg1 mutant. In a wild type strain, FBPase was degraded within a 2 h chase period, whereas FBPase degradation was significantly retarded in the Δreg1 strain (Fig. 1B).

**REG1 Does Not Prevent FBPase Phosphorylation**—Reg1p is known to play an important role in signal transduction pathways (36, 37, 46). In wild type cells, FBPase is phosphorylated following a shift from low glucose to high glucose containing media via a Ras2p and cAMP-mediated signaling pathway (45). At present, however, it is unknown whether phosphorylation plays a direct role in FBPase trafficking through the Vid vesicle pathway. To test whether the absence of the REG1 gene affects the ability of cells to phosphorylate FBPase in response to glucose, Δreg1 cells were metabolically labeled with 32P and then shifted to glucose-containing media for various periods of time. Cell lysates were subjected to immunoprecipitation analysis to determine the level of FBPase phosphorylation. As controls, we utilized a RAS2 strain and a ras2Δ1185 strain, the latter of which has been shown to exhibit a drastic reduction in FBPase phosphorylation (45). As shown in Fig. 2A, the RAS2 strain exhibited a high level of FBPase phosphorylation following a glucose shift, whereas the ras2Δ1185 strain had a much lower level of phosphorylated protein. The Δreg1 strain, on the other hand, had levels of phosphorylation that were as high, or somewhat higher than wild type cells (Fig. 2B). Note that the overall levels of FBPase were similar for each of these strains, indicating the differences were not due to changes in the levels of this protein. Because FBPase phosphorylation was not prevented in the absence of Reg1p, Reg1p is unlikely to be involved in the signaling pathway leading to FBPase phosphorylation.

**Reg1p Is Not Required for FBPase Import to Vid Vesicles**—The trafficking of FBPase to the vacuole requires the presence of functional Vid vesicles. Although Vid vesicle formation is compromised in the absence of the UBC1 gene (17), this is the only gene that is known to be essential for this process. To determine whether Reg1p plays a role in Vid vesicle formation, we performed a differential centrifugation analysis using Vid24p as a specific marker of Vid vesicles. Cells were starved and shifted to glucose-rich media for 30 min, and cell extracts were subjected to differential centrifugation as described previously (16, 18). As is shown in Fig. 3A, the levels of Vid24p in the Vid vesicle fraction were reduced in the Δubc1 strain. In contrast, similar amounts of Vid24p were detected in the Vid vesicle fractions in both wild type and Δreg1 strains, suggesting that REG1 does not affect Vid vesicle formation.

In wild type cells, FBPase is imported into Vid vesicles following a glucose shift (14–16). Therefore, we examined whether FBPase import was compromised in the absence of the REG1 gene. Differential centrifugation experiments were performed using lysates obtained from Δreg1, Δvid24, and Δubc1 cells that had been shifted to glucose (Fig. 3B). A substantial portion of FBPase was found in the Vid vesicle-containing...
fraction of Δreg1 and Δvid24 cells. By contrast, low levels of FBPase were found in the Vid vesicle fraction of the Δubc1 mutant. Thus, this suggests that FBPase can be imported into Vid vesicles in the absence of Reg1p.

Reg1p Is Involved in the Trafficking of Vid Vesicles to the Vacuole—To determine whether Reg1p plays a role in the second step of the FBPase pathway, we used an in vitro assay to quantify this process (21). For this assay, FBPase was fused with a truncated form of alkaline phosphatase that lacks the N-terminal 60 amino acids. Vid vesicles containing FBPase-Δ60Pho8p can be isolated and combined with isolated vacuoles and cytosol from cells lacking the PHO8 gene. After incubation, the activation of alkaline phosphatase can be used to quantitate Vid vesicle-vacuole fusion in vitro. Furthermore, this assay can be used to identify the function site of a particular protein, because various combinations of mutant or wild type Vid vesicles, vacuoles, or cytosol can be examined.

The Δreg1 strain exhibited a very low level of FBPase-Δ60Pho8p expression following transformation. Because the in vitro assay relies upon high level expression of the fusion protein, we were unable to accurately assess Vid vesicle function in the Δreg1 strain. However, we were able to examine the function of Δreg1 vacuoles and cytosolic components. For these assays, Vid vesicles were isolated from a wild type strain expressing high levels of FBPase-Δ60Pho8p. When wild type Vid vesicles, vacuoles, and cytosol were tested in the in vitro assay, alkaline phosphatase activity was high, indicating that the fusion protein had been processed by the vacuole Pep4p (Fig. 4A). In contrast, when Δreg1 vacuoles were used, alkaline phosphatase activity was reduced, suggesting Reg1p plays a role on the vacuole. However, cytosol from the Δreg1 mutant did not reduce the alkaline phosphatase activity significantly.

Because Reg1p exerts its function on the vacuole, we tested whether this protein could be found in the vacuole fraction. Initially, we used Reg1p-GFP and found that the GFP signal was primarily in the cytosol, consistent with a previous report (37). Because cytosolic distribution could mask the fluorescence signal in the vacuole, we used differential centrifugation techniques to separate these compartments (18). Using this procedure, the vacuole marker Vph1p is enriched in the 13,000 g pellet fraction, whereas the cytosol marker enolase is enriched in the 200,000 × g supernatant fraction. The Vid vesicle marker Vid24p is found primarily in the 100,000 × g pellet fraction, with a smaller amount in the 200,000 × g pellet fractions (18). As is shown in Fig. 4B, Reg1p was present in multiple fractions. The soluble fraction S200 contained the highest amounts of Reg1p, whereas a significant amount of Reg1p was found in the vacuole-enriched fraction (P13). A small amount of Reg1p was also present in the Vid vesicle-enriched fraction (P100 and P200).
A Role for Glc7p/Reg1p in Vid Vesicle Trafficking

Vid Vesicles and Vacuole Functions Require Glc7p—Reg1p is known to interact with the type I phosphatase Glc7p both genetically and biochemically (34, 36). GLC7 is an essential gene that is involved in a number of cellular processes, including glucose repression, glycerogen metabolism, translation, sporalization, chromosome segregation, and cell cycle progression (26–31, 34–37, 47–54). Furthermore, different Glc7p functions are mediated through its interaction with distinct regulatory subunits (26–31, 34–37). To assess whether the interaction of Reg1p with Glc7p plays a role in the trafficking and degradation of FBPase, we used a strain with the gclc7-T152K mutation. This mutation has been shown to interfere with the Glc7p–Reg1p interaction, as determined by yeast two hybrid analysis (36). However, the interaction characteristics under our in vivo conditions have not been examined. To verify this defect, wild type and gclc7-T152K strains expressing Reg1p-V5-His6 were glucose-starved and shifted to fresh glucose for 30 min. Reg1-V5-His6 was pulled down with nickel beads, and bound proteins were immunoblotted with anti-V5 and Glc7p antibodies (Fig. 5A). When a wild type strain was examined, we observed the interaction of Reg1p and Glc7p, as reported previously (36). In contrast, the level of this complex was substantially reduced when the same experiments were performed using the gclc7-T152K mutant strain. The reduced interaction was not due to a decrease in expression of Reg1p and Glc7p, because similar levels of these proteins were found in total lysates from these strains (Fig. 5B).

We next tested FBPase degradation in the gclc7-T152K strain. This strain exhibited a retarded degradation (Fig. 5C), suggesting that the Glc7p–Reg1p complex plays some role in this process. Because different mutations of GLC7 have been shown to affect distinct biological processes (36, 43, 54), we attempted to determine whether the FBPase degradation defect is allelic specific. For instance, the gclc7-T152K mutation inhibits the glucose repression function of the protein, but it does not impair glycerogen synthesis (36, 54). In contrast, the gclc7-1 mutation impairs the binding to Gac1p leading to a defective glycogen synthesis (36, 46, 54). We found that the gclc7-1 strain had minimal defect in FBPase degradation (Fig. 5C).

Reg1p is known to interact with a number of proteins in addition to Glc7p. For example, Snf1p is the yeast homologue of the catalytic subunit of AMP-activated protein kinase and is shown to phosphorylate Reg1p (55). Reg1p also interacts with Snf4p, the activating subunit of the Snf1p kinase complex (55). In this study, neither the Δsnf1, nor the Δsnf4 mutation inhibited FBPase degradation to a significant degree (Fig. 5D), suggesting that Snf1p, Snf4p, and/or phosphorylation of Reg1p by the Snf1p complex are not critical for FBPase degradation. Reg1p can also interact with Bmh1p and Sip5 (56). However, FBPase was degraded in strains containing the deletion of these genes (Fig. 5D), suggesting that they are not involved in the FBPase degradation pathway.

If the Glc7p–Reg1p complex is required for FBPase degradation, then Glc7p and Reg1p most likely function in the same step of the FBPase degradation pathway. Therefore, the role of Glc7p in Vid vesicle trafficking was tested using our in vitro fusion assay. The gclc7-T152K mutant expressed high levels of FBPase-Δ60Poph8 and allowed us to examine Vid vesicles in this strain. As is shown in Fig. 6A, alkaline phosphatase activity was low, when Vid vesicles or vacuoles from the gclc7-T152K mutants were used. In contrast, alkaline phosphatase activity remained high when mutant cytosol was used. This suggests that Glc7p functions predominantly on Vid vesicles, although it is also required for vacuole function to a lesser extent.

If Glc7p functions on the vacuole or Vid vesicles, this protein may associate with these organelles. As is shown in Fig. 6B, the highest amount of Glc7p was found in the cytosolic enriched fraction. Lower amounts of Glc7p were also found in the vacuole- and Vid vesicle-enriched fractions. Because the majority of Reg1p and Glc7p is cytosolic, the localization of Reg1p (Fig. 4B)
and Glc7p in the vacuole and Vid vesicle fraction may result from nonspecific binding of these proteins to these organelles. However, the presence of Glc7p in the vacuolar enriched fraction was consistent with a previous report in which Glc7p was found in this organelle and plays a role in homotypic vacuole fusion (22).

**Shp1p and Glc8p Are Necessary for Vid Vesicle and Vacuole Functions**—Glc7p is known to regulate a diverse variety of processes, and this is mediated via binding to different regulatory subunits. As mentioned above, Glc7p is targeted to glycogen accumulation processes by interacting with Gac1p and Pig1p (26, 27). Reg2p, on the other hand, targets Glc7p to growth and cell cycle progression (28), whereas Gip1p regulates meiosis and sporulation (31). In addition, Bud14p directs Glc7p to cellular morphogenesis (30), whereas Bni4p targets Glc7p to cytokinesis (29). When genes encoding these proteins were deleted, FBPase degradation was not impaired (Fig. 7A). Thus, these Glc7p regulatory proteins are not required for FBPase degradation. In addition to the aforementioned Glc7p-interacting proteins, Shp1p and Glc8p are known to regulate the phosphatase activity of Glc7p (38, 39). Accordingly, mutations or deletions of these genes reduce the phosphatase activity of Glc7p (38, 39). When FBPase degradation was examined in the Δglc8 and Δshp1 strains, it was retarded (Fig. 7B).

The FBPase degradation defects in the Δglc8 and Δshp1 strains were not due to a decrease in the Glc7p-Reg1p interaction. When Reg1p was immunoprecipitated from wild type, Δglc8, and Δshp1 strains, the amounts of bound Glc7p were comparable (Fig. 8). Likewise, changes in the subcellular distribution or expression of Reg1p or Glc7p were not responsible for this defect, because these parameters were similar to those seen in wild type cells (data not shown). Therefore, in these mutants, reduced Glc7p phosphatase activity appears to be the cause of the FBPase degradation defect.

We next examined whether the Δglc8 and Δshp1 mutants affected the same step of the FBPase degradation pathway as Reg1p and Glc7p. Via the use of our in vitro fusion assay, we found that Vid vesicles were severely defective, whereas the vacuole was partially defective in these mutants (Fig. 8, C and D). These results further confirm that Glc7p and its regulators play an essential role in the trafficking of Vid vesicles to the vacuole. Furthermore, they all exert their functions primarily on Vid vesicles, and to a lesser degree on the vacuole.
Figure 5. The \textit{glc7-T152K} mutation reduces \textit{Reg1p-Glc7p} interaction and inhibits FBPase degradation. \textbf{A}, the interaction of \textit{Reg1p} with \textit{Glc7p} was examined in wild type and \textit{glc7-T152K} strains expressing \textit{Reg1p-V5-His6}. \textit{Reg1p-V5-His6} was pulled down by nickel beads from both transformed and untransformed cells, and the bound \textit{Glc7p} was examined by Western blotting with \textit{Glc7p} antibodies. \textbf{B}, total lysates from these cells were examined for the expression of \textit{Reg1p} and \textit{Glc7p} by Western blot. \textbf{C}, the wild type, \textit{glc7-T152K}, and \textit{glc7-1} mutant strains were shifted from low to high glucose media and examined for FBPase degradation. \textbf{D}, the degradation of FBPase was tested in \textit{Δsnf1}, \textit{Δsnf4}, \textit{Δbmh1}, and \textit{Δsip5} strains.
DISCUSSION

We have utilized a variety of methods to identify proteins that play a role in the degradation of FBPase. Genetic screens have yielded a number of VID genes that are required for the targeting of FBPase to the vacuole following a shift to glucose rich media (57). Furthermore, via the use of in vivo and in vitro assays, we have been able to identify the site of action of the VID gene protein products (14–21). However, we have not identified any FBPase-interacting proteins that may play a role in early steps of the FBPase degradation pathway. Therefore, to address this issue, we screened for GST-tagged proteins that might exhibit an interaction with FBPase. Here we hypothesized that FBPase-interacting proteins may regulate FBPase recognition or FBPase sequestration into Vid vesicles. Unfortunately, we were unable to identify any FBPase-binding proteins that carried out these functions. However, we did discover a previously undefined role for the phosphatase Reg1p-Glc7p in the Vid vesicle trafficking pathway.

Although FBPase degradation was compromised in the absence of the REG1 gene, we showed that Reg1p was not involved in any of the early steps of the FBPase degradation pathway. For instance, the absence of REG1 did not affect the ability of cell to phosphorylate FBPase in response to glucose. In addition, Vid vesicle biogenesis was not compromised in cells lacking the REG1 gene, and Vid vesicles were functional in terms of importing FBPase. These results appear to be inconsistent with the idea that there is a functional FBPase-Reg1p interaction in cells. Accordingly, when we attempted to verify this interaction under our in vivo conditions, we did not observe a significant interaction between Reg1p and FBPase in wild type, glu7-T152K, Δshp1, or Δgln8 strains (data not shown). Little interaction was observed when Reg1p was immunoprecipitated and then blotted with FBPase antibodies or when FBPase was immunoprecipitated and examined for the presence of bound Reg1p. One possibility for this discrepancy may be that the GST-Reg1p fusion protein has an altered conformation that allows artificial binding of FBPase that does not normally occur under in vivo conditions. Alternatively, this may indicate that the interaction is too weak to capture using our standard immunoprecipitation conditions. However, under the same conditions, an interaction between Reg1p and Glc7p was observed.

Reg1p is part of the Glc7p protein phosphatase complex, and this complex plays a required role in a number of cellular processes (28, 32–37, 47, 48, 54). Glc7p has important functions in homotypic vacuole fusion, endoplasmic reticulum-to-Golgi transport, and endocytic vesicular trafficking (22). For homotypic fusion, Glc7p is required for the last fusion step, although the PP1 substrate proteins have not been identified. Following docking of vacuoles, calcium is released from the vacuole and this calcium efflux stimulates calmodulin binding to the vacuole H⁺-ATPase. This then triggers the formation of a transcomplex on opposing vacuoles (22, 58). In this present study, we have demonstrated a requirement for GLC7 in what is most likely a membrane fusion event: Vid vesicle to vacuole trafficking. This conclusion was based upon the use of mutant strains that interfered with Reg1p interactions. However, corroborative results were also obtained with strains in which the Glc7p...
regulator genes SHP1 and GLC8 were deleted. The absence of these genes resulted in reduced phosphatase activity of Glc7p and a defect in FBPase degradation and trafficking.

For homotypic vacuole fusion, Glc7p is found in a multisubunit complex containing calmodulin in the vacuole-enriched fraction (22). Furthermore, Glc7p is also present in the vertex that contains Vam3p, Ypt7p, homotypic fusion vacuole protein sorting, and Vac8p (58). Consistent with these results, we observed a portion of Glc7p in the vacuole fraction. Likewise, a small, but detectable amount of Glc7p and Reg1p can also be found in the Vid vesicle fraction. Because the majority of these proteins are in the cytosol, we cannot rule out the possibility that nonspecific binding of Reg1p-Glc7p to these organelles occurred under our conditions. It will be important to address the questions whether Reg1p-Glc7p binding to these compartments is specific and whether stable or transient binding is necessary for Vid vesicle/vacuole fusion. Mutations that interfere with Reg1p or Glc7p binding to these organelles will be useful for these studies in the future.

Multiple glc7 mutations are available, with many showing unique characteristics in terms of their physiological characteristics (36, 42, 43, 54). Therefore, we tested several glc7 mutants that have different phenotypic effects (22, 43). The glc7-109, glc7-127, and glc7-133 mutants were not defective in FBPase degradation, whereas glc7-132 and glc7-10 mutants were partially defective (data not shown). These results suggest that the FBPase degradation defect was allelic-specific.

Whether these mutations affect Glc7p binding to different regulatory subunits has not been established. For this reason, we chose two mutations that are known to interfere with the interactions between Glc7p and two well characterized regulatory subunits, Gac1p and Reg1p. The glc7-1 mutant interferes with Gac1p binding but not Reg1p binding. Interestingly, this mutant strain was not defective in FBPase degradation. In contrast, the glc7-T152K point mutation that reduces the interaction between Glc7p and Reg1p did adversely affect FBPase degradation. Thus, we suspect that Reg1p specifically targets Glc7p for function in the Vid vesicle-vacuole trafficking pathway. When we examined deletion mutant strains of several other Glc7p regulatory subunits, they were not defective in FBPase degradation, suggesting that they are not involved in the FBPase degradation pathway. These results and our previous observations suggest that a specific interaction between Reg1p and Glc7p is important for FBPase degradation to occur.

Vid vesicles are thought to fuse with vacuoles via mechanisms that are similar to other vesicular fusion events. Vid vesicle-vacuole fusion requires the participation of a number of SNARE proteins that are also necessary for homotypic vacuolar fusion (21). However, Vid vesicle/vacuole fusion appears to more closely resemble heterotypic rather than homotypic fusion. A number of v-SNAREs and t-SNAREs are present on both Vid vesicles and vacuoles. However, the v-SNAREs (Ykt6p, Nyv1p, and Vti1p) are required on the vesicles, whereas the t-SNAREs Vam3p only functions on the vacuole.
Although the role of Reg1p on Vid vesicles was not tested in this study due to technical difficulties, Glc7p, Shp1p, and Glc8p are all required on Vid vesicles, because Vid vesicles are the most defective components in these mutants. Furthermore, mutations of these genes all reduced the functions of vacuoles albeit to a lesser extent, suggesting that they are also required for vacuoles to function fully. At the present time, exactly how Glc7p regulates Vid vesicles/vacuole fusion is not known. It is established that Glc7p controls the lipid bilayer mixing step of the Vid vesicles/vacuole fusion event, similar to that described for homotypic vacuole fusion. It remains to be determined whether this process also requires the efflux of calcium or the action of calmodulin, as has been shown for homotypic vacuole fusion. Likewise, it has not been established whether the same protein substrates mediate the effects of Glc7p on these two different processes. Further experiments will be required to identify substrate proteins that are regulated by the Reg1p-Glc7p phosphatase and to determine their roles in the delivery of the Vid vesicle cargo to the vacuole.

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The Type 1 Phosphatase Reg1p-Glc7p Is Required for the Glucose-induced Degradation of Fructose-1,6-bisphosphatase in the Vacuole
Dong-Ying Cui, C. Randell Brown and Hui-Ling Chiang

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