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Identification of Receptor-like Protein for Fructose-1,6-bisphosphatase on Yeast Vacuolar Membrane

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In yeast the key gluconeogenic enzyme, fructose-1,6-bisphosphatase (FBPase), is selectively targeted from the cytosol to the lysosome (vacuole) for degradation when glucose starved cells are replenished with glucose. The pathway for glucose induced FBPase degradation is unknown. To identify the receptor-mediated degradation pathway of FBPase, we investigated the presence of the FBPase receptor on the vacuolar membrane by cell fractionation experiments and binding assay using *vid* mutant (vacuolar import and degradation), which is defective in the glucose-induced degradation of FBPase. FBPase sedimented in the pellets from *vid24-1* mutant after centrifugation at 15,000 × g for 15 min, suggesting that FBPase is associated with subcellular structures. Cell fractionation experiments revealed that FBPase is preferentially associated with the vacuole, but not with other organelles in *vid24-1*. FBPase enriched fractions that co-fractionated with the vacuole were sensitive to proteinase K digestion, indicating that FBPase is peripherally associated with the vacuole. We developed an assay for the binding of FBPase to the vacuole. The assay revealed that FBPase bound to the vacuole with a Kd of 2.3×10^{6} M. The binding was saturable and specific. These results suggest that a receptor for FBPase degradation exists on the vacuolar membrane. It implies the existence of the receptor-mediated degradation pathway of FBPase by the lysosome.

Keywords: FBPase receptor, Fructose-1,6-bisphosphatase, Protein degradation, Vacuolar degradation pathway, *vid24-1* mutant.

Introduction

Protein synthesis and degradation are strictly regulated to allow rapid adaptation of living cells to the environment. Protein degradation in mammalian cells and yeast cells is accomplished *via* two main routes; the proteasomal pathway and the lysosomal/vacuolar pathway. The proteasomal pathway is primarily involved in the degradation of ubiquitinated and short-lived proteins (Finley and Varshavsky, 1985; Jentsch *et al.*, 1990); whereas the lysosomal/vacuolar pathway is responsible for the degradation of long-lived proteins, organelles and macromolecules (Dice, 1990; Dunn, 1990; Seglen *et al.*, 1990).

Lysosomal degradation of macromolecules can be achieved through endocytosis, crinophagy, non-selective autophage, a heat shock protein mediated transport or a receptor protein mediated transport pathway (Chiang *et al.*, 1989; Dunn, 1990; Seglen *et al.*, 1990; Terlecky *et al.*, 1992; Cuervo *et al.*, 1994; Cuervo *et. al.*, 1996). In yeast *Saccharomyces cerevisiae*, the α-factor is internalized by receptor mediated endocytosis and delivered to the vacuole for degradation (Chvatchko *et al.*, 1986; Raths *et al.*, 1993; Munn and Riezman, 1994). Non-selective macroautophage of organelles and cytosolic proteins by the vacuole occurs under the stress of nitrogen starvation (Takeshiege *et al.*, 1992; Tsukada and Ohsumi, 1993). Whether or not crinophage or carrier-mediated transport exists in yeast still needs to be studied. The receptor protein for the selective uptake of RNase A and glyceraldehyde-3-phosphate dehydrogenase by lysosomes was identified as the lysosomal glycoprotein LGP96 (Cuervo and Dice, 1996). Overexpression of LGP96 increases the activity of the selective lysosomal degradation pathway both *in vivo* and *in vitro* (Cuervo and Dice, 1996).

In *S. cerevisiae*, fructose-1,6-bisphosphatase (FBPase), the key gluconeogenic enzyme is targeted to the vacuole for degradation when glucose-starved cells are replenished with glucose (Chiang and Schekman, 1991). Glucose also induces targeting of peroxisomes to the vacuole for degradation. Peroxisomes are internalized by autophagy that leads to the destruction of the whole organelle in the vacuole. Upon re-growth of cells on the glucose, the galactose transporter (Gal2p) is also delivered from the plasma membrane to the vacuole *via* endocytosis for degradation. The pathway for

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FBPase degradation by glucose in *S. cerevisiae* has not yet been investigated. Targeting of FBPase, peroxisomes and Gal2p to the vacuole for degradation all require the synthesis of new proteins, suggesting the existence of the receptor protein (Chiang and Schekman, 1991).

Yeast *vid* mutants (vacuolar import and degradation) that are defective in the glucose-induced degradation of FBPase have been developed by genetic screening (Hoffman and Chiang, 1996). Among those mutants *vid24-1* displays preferential staining of FBPase on the vacuolar membrane when stained using immunofluorescence (Ko and Chiang, unpublished data). However, FBPase could not be internalized into the vacuole for degradation (Chiang and Chiang, 1998). This suggests that FBPase binds to the vacuolar membrane, possibly through the receptor protein and is degraded by a receptor mediated degradation pathway.

In this study, we investigated the presence of a FBPase receptor on the vacuolar membrane by cell fractionation experiments and binding assay. Cell fractionation studies showed that FBPase was present in the vacuolar fraction and was also sensitive to proteinase K digestion. This suggests the existence of a receptor-like protein on the surface of the vacuolar membrane. Binding assay that used radiolabeled FBPase revealed the existence of specific binding sites on the vacuolar membrane. These results demonstrate for the first time the existence of the FBPase receptor on the vacuolar membrane and support the existence of a receptor-mediated degradation pathway of FBPase by yeast lysosome.

Materials and Methods

Materials All chemicals were of reagent grade. Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, fructose-1,6-bisphosphate (FBP), and AMP were purchased from Sigma Chemical Co (St. Louis, USA). [^{35}S]methionine was purchased from NEN Life Science Products (Boston, USA). Yeast extract and peptone were purchased from Difco Laboratories Inc. (Detroit, USA). Lyticase was prepared as described by Scott and Schekman (1980).

Strains, media and antibodies Yeast strain *vid24-1* (*MATα trp1 ade2 his3-Δ200 ura3-52 leu2,3-112 vid24-1*) was used in this study. The YPD medium contained 1% yeast extracts, 2% peptone and 2% glucose. The YPKG medium contained 1% yeast extracts, 2% peptone, 1% potassium acetate and 0.5% glucose. The synthetic medium contained 6.7 g/l of a yeast nitrogen base that lacked both amino acids and ammonium sulfate that was supplemented with 2% glucose and 1.8 g/l amino acid mix without *ura* and *met*. An antibody directed against FBPase was generated against the purified FBPase.

Cell fractionation The *vid24-1* was grown in a YPKG medium for 48 h at 30°C, transferred to a YPD medium, and grown further for 60 min. Spheroplasts were prepared as follows: Cells were resuspended in 20 mM Hepes-KOH (pH 9.4), 40 mM EDTA and 100 mM β-mercaptoethanol, and gently mixed for 30 min at 30°C; the cells were centrifuged at 1,500 × g for 10 min, resuspended in lyticase solution (5 units/OD) containing 1.5 M sorbitol, 10 mM Tris-HCl (pH 7.5), and incubated for 30 min at 30°C. The spheroplasts were centrifuged at 1,500 × g for 10 min, resuspended in 1M sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes-KOH (pH 6.8), and the spheroplast pellets were frozen in liquid nitrogen and stored at -80°C for later use.

Cell fractions were prepared as follows: The spheroplasts were resuspended in 2 ml of TEA buffer (10 mM triethanolamine-acetic acid pH 7.5, 0.8 M sorbitol, 1 mM EDTA) containing protease inhibitors (100 μg/ml of PMSF, 50 μg/ml of leupeptin and 50 μg/ml of aprotinin) and homogenized 20-25 times in a Dounce homogenizer. The lysate was centrifuged at 500 × g for 5 min and the unlysed spheroplasts were re-extracted in 2 ml of the same buffer. The combined post nuclear supernants (S1) were centrifuged at 15,000 × g for 15 min in order to obtain the medium speed pellet (P2) and supernant (S2). The P2 fraction was resuspended in 1 ml of TEA buffer and was loaded on top of the sucrose gradient, which consisted of 2 ml of 61%, 3.5 ml of 37%, 2 ml of 29%, 1 ml of 27% and 1.5 ml of 22% (wt/vol) sucrose. The gradient was subjected to centrifugation at 100,000 × g for 2 h and 0.85 ml fractions were collected from the top.

Proteinase K digestion Samples from each fraction were treated with 100 μg/ml of proteinase K for 15 min on ice and the reaction was terminated with 10% trichloroacetic acid (TCA). Proteins were separated on SDS-PAGE gels. FBPase was detected by immunoblotting with anti-FBPase antibody and analyzed by densitometry.

Enzymatic assays Activity assays for glucose-6-phosphate dehydrogenase, NADPH cytochrome C reductase, vacuolar α-mannosidase were performed as described by Roberts (Roberts *et al.*, 1991). Assays for carboxypeptidase Y (CPY) activity (Roberts *et al.*, 1991), plasma membrane ATPase activity (Serrano, 1983), and GDPase activity (Abeijion *et al.*, 1989) were performed as described.

Preparation of radiolabeled FBPase Radiolabeled FBPase was prepared according to the methods as described previously from a yeast strain containing FBPase expression plasmid AU125 (Rogers *et al.*, 1988). The yeast was grown at 30°C in 1 liter of synthetic medium containing 10 mCi of [^{35}S]methionine. The cells were harvested by centrifugation at 1,500 × g for 10 min, washed with cold deionized water, and resuspended in 1 volume of 2 mM EDTA (pH 7.0) and 0.12 mg/ml of PMSF. The cell suspension was mixed with 2 volumes of prechilled glass beads (diameter 0.5 mm) and the cells were disrupted by vigorous vortexing for three 1 min periods with a 5 min pause between treatments. The homogenate was drawn off the beads, and the beads were rinsed with 0.5 volumes of 2 mM EDTA containing 0.12 mg/ml of PMSF. The combined supernatants were centrifuged at 25,000 × g for 25 min. Then the supernant was adjusted to pH 7.5 with a Tris base. Magnesium sulfate was added to a final concentration of 5 mM and the solution was allowed to stand for 60 min at room temperature. The solution was adjusted to 18 mM FBP and 1.8 mM AMP by the addition of a 1/10

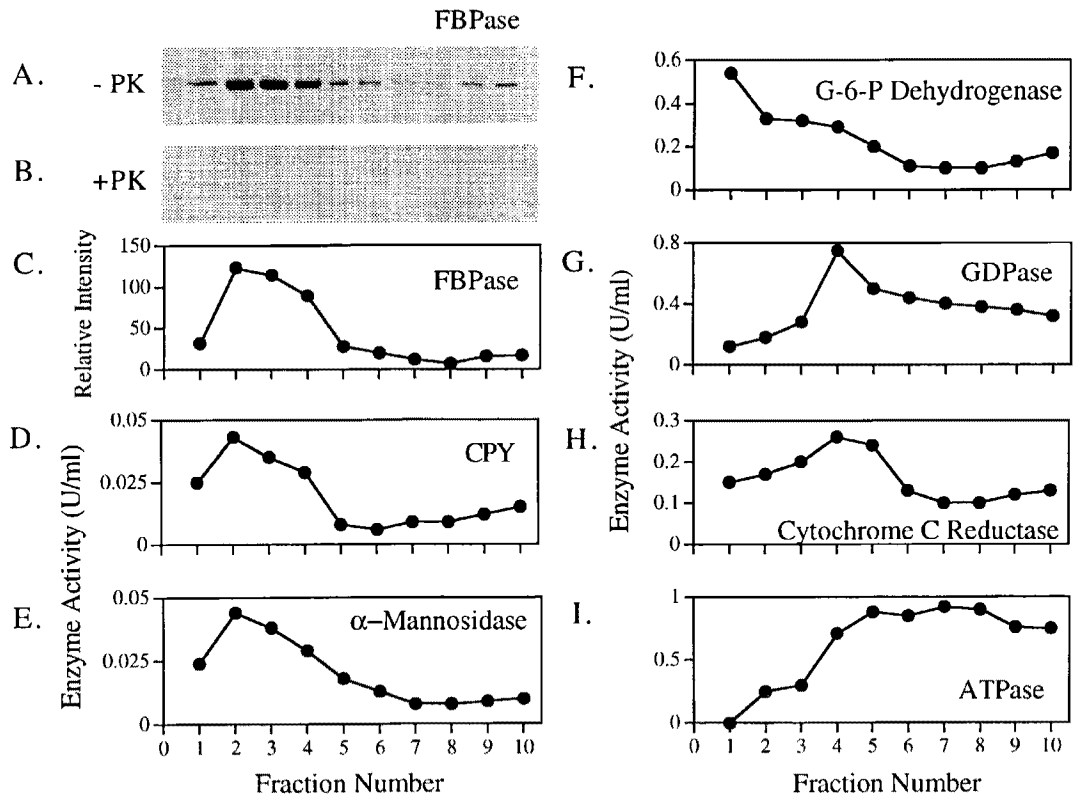

Fig. 1. FBPase is preferentially associated with the vacuole in the yeast strain *vid24-1*. Yeast mutant defective in FBPase degradation, *vid24-1*, was grown in a YPKG medium and transferred to glucose for 60 min. Cell fractionation was performed as described in the 'Materials and Methods'. Samples were collected from 20-40% sucrose gradient centrifugation. FBPase was detected by immunoblotting (A and B), with (B) or without (A) the addition of proteinase K, and analyzed by densitometry (C). Various markers were used to localize subcellular organelles: CarboxypeptidaseY for vacuolar soluble proteins (D), α-mannosidase for vacuolar membrane proteins (E), glucose-6-phosphate dehydrogenase for cytosolic proteins (F), GDPase for Golgi apparatus proteins (G), NADPH cytochrome C reductase for endoplasmic reticulum proteins (H), and plasma membrane ATPase for plasma membrane proteins (I). All enzyme assays were performed as described in the 'Materials and Methods'. Data are representatives of at least three independent experiments.

volume of 200 mM FBP and 20 mM AMP mixture, and then was immediately heated for 3 min 30 s at 67°C. The mixture was cooled on ice and centrifuged at 25,000 × g for 15 min. Ammonium sulfate (0.34 g/ml) was added to the supernatant and stirred for 45 min at 4°C. After centrifugation at 25,000 × g for 40 min, the pellet was resuspended in 3 ml of a buffer containing 0.4 M sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes-KOH, pH 6.8 and 25% glycerol, and dialyzed for 16-20 h against 1 liter of the same buffer. The dialyzed FBPase was stored at -80°C and used for the binding assay. The protein concentration of dialyzed FBPase was determined by the Lowry Method (Lowry *et al.*, 1951).

Isolation of vacuoles A derivative of *vid24-1* in which the FBPase gene was knocked out was used for the isolation of vacuoles. The yeast was grown in a YPKG medium for 48 h at 30°C, transferred to a YPD medium, and grown further for 60 min. Cells were spheroplasted and fractionated as described above in the cell fractionation. The pellet (P2) that contains the vacuoles was separated on a 20-40% sucrose gradient (13 ml) and 0.85 ml fractions were collected from the top. The second and the third fractions were combined and used for the binding assay.

***In vitro* binding assay** The mixture for the *in vitro* binding assay contained the following reagents in 0.2-0.6 ml of final volume: 100 μg of isolated vacuoles, 0.5 μCi of [^{35}S]FBPase, 20 mM Hepes-KOH (pH 6.0), 0.4 M sorbitol, 150 mM potassium acetate, and 5 mM magnesium acetate. The mixture was incubated for 60 min at 4°C and the binding was terminated by centrifugation at 13,000 × g for 10 min to pellet the vacuoles. The vacuole pellets were washed with a buffer containing 20 mM Hepes-KOH (pH 6.0), 0.4 M sorbitol, 150 mM potassium acetate, and 5 mM magnesium acetate followed by centrifugation at 13,000 × g for 10 min. The washing process was repeated three times. Then pelleted vacuoles were resuspended in 50 μl of the same buffer and the radioactivity was determined using a Beckman liquid scintillation counter.

Results

Subcellular distribution of FBPase in *vid24-1* mutant In order to determine the distribution of FBPase in yeast cells, a cell fractionation experiment was performed as described in the Methods. Fig. 1 shows the separation of various organelles on the sucrose gradient from *vid24-1* cells that were

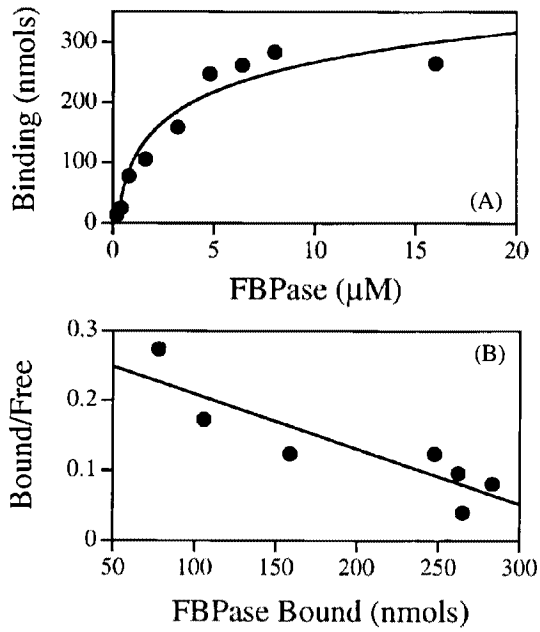

Fig. 2. The binding of FBPase to the vacuole is saturable and specific. Vacuoles were isolated from *vid24-1*, as described in the 'Materials and Methods'. (A) Isolated vacuoles (100 μg) were incubated under standard conditions with increasing amounts of FBPase for 60 min at 4°C. Values for the non-specific association of FBPase to vacuoles were subtracted from the total values. (B) Scatchard plot of (A). Data are representatives of three independent experiments.

replenished with glucose for 60 min. The fractionation procedure produced enriched vacuoles, which could be effectively separated away from most subcellular compartments such as the plasma membrane, secretory organelles and the cytosol. Enzymatic assays with the marker enzymes for vacuoles, CPY and α-mannosidase showed that the vacuoles were enriched in fraction 2 (Fig. 1D and Fig. 1E). Fig. 1F shows that the cytosolic marker, glucose-6-phosphate dehydrogenase remained on the top of the gradient in fraction 1. The endoplasmic reticulum (ER) protein, cytochrome C reductase peaked at fraction 4 (Fig. 1H). It co-fractionated with the Golgi apparatus marker GDPase (Fig. 1G). The plasma membrane ATPase showed a broader distribution from fraction 5 to fraction 9 (Fig. 1I).

As shown in Fig. 1A, FBPase was enriched in fraction 2, 3 and 4. A quantitative analysis by densitometry indicated that FBPase peaked at fraction 2 (Fig. 1C). The FBPase co-fractionate with CPY (Fig. 1D), as well as α-mannosidase (Fig. 1E), demonstrated that FBPase was co-present with the vacuoles.

Whether FBPase was peripherally associated with the vacuole, or embedded in the vacuolar membrane, was examined by the proteinase K treatment. As shown in Fig. 1B, FBPase was completely digested with proteinase K and not detectable by Western blot analysis. The results suggest that FBPase is peripherally associated with the vacuolar membrane.

These results demonstrate that FBPase is associated with the vacuole, but not with the ER, Golgi, cytosol, or plasma membrane in *vid24-1*. It also demonstrates that FBPase is peripherally associated with the vacuoles.

FBPase binding to the vacuole was saturable and specific

Kinetic studies for FBPase binding to the vacuoles were performed using the vacuolar fractions from a FBPase deficient mutant of *vid24-1*, as described in the Materials and Methods. The fractions 2 and 3 from the sucrose gradient as in Fig. 1 represent vacuole-enriched fractions. The vacuolar fractions used in the binding study were essentially the same as in Fig. 1 except that the vacuolar fractions lack an endogenous FBPase. The mixtures containing the vacuoles and ^{35}S-labeled FBPase were incubated and, at timed intervals, the reactions were terminated by centrifugation that separated

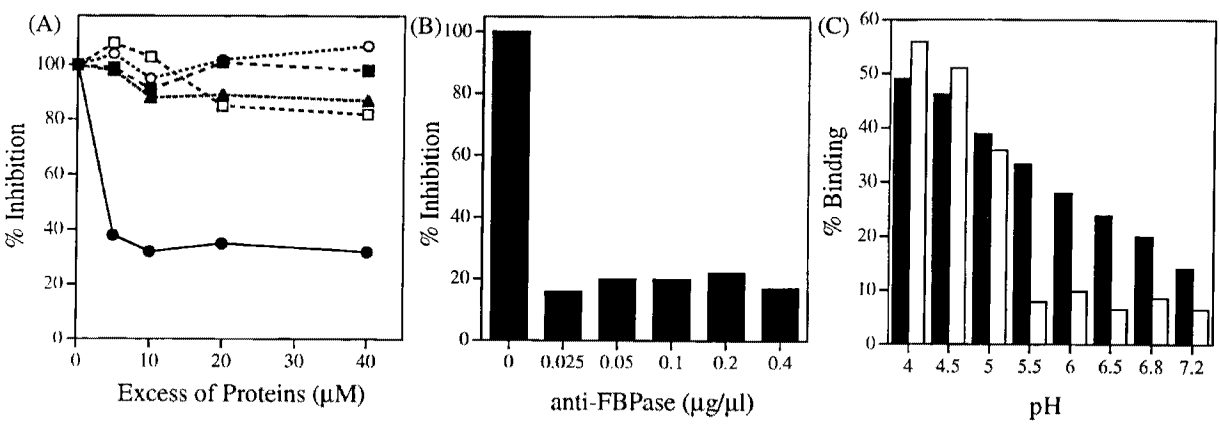

Fig. 3. Effects of cytosolic proteins, anti-FBPase antibody and pH on the binding of FBPase to the vacuole. The isolated vacuoles (100 μg) from *vid24-1* were incubated for 60 min at 4°C (A) in the absence or presence of excess amounts (50-fold of labeled FBPase) of unlabeled FBPase (●), RNase A (■), glyceraldehyde-3-phosphate dehydrogenase (❑), BSA (▲) and ovalbumin (○) (B) in the absence or presence of anti-FBPase antibody, and (C) in the absence (closed bar) or presence (open bar) of excess unlabeled FBPase under the indicated pH value. Data are representatives of three independent experiments.

the pellets containing FBPase bound to the vacuole from the supernant containing unbound FBPase. FBPase binding was determined by counting the radioactivity associated with the vacuole.

FBPase binding to the vacuole steadily increased the first 45 min and reached a plateau at 60 min (data not shown). To determine the specificity and saturation of the binding, we incubated vacuoles with increasing amounts of radiolabeled FBPase for 60 min on ice. Total binding was determined in the absence of unlabeled FBPase. Specific binding was determined by subtracting the value for non-specific binding from the values for total binding. The values for non-specific binding were determined in the presence of 50-fold unlabeled FBPase. Fig. 2 shows that FBPase binding to the vacuole was saturable. The binding was specific in that it could be competed with excess amounts of unlabeled FBPase. Scatchard plot analysis indicated that FBPase bound to the vacuole with a Kd of 2.3×10^{-6} M.

Effects of cytosolic proteins, anti-FBPase antibody and pH on the binding of FBPase to the vacuole Whether or not ubiquitous cytosolic proteins, and the proteins that are known to be degraded by a receptor mediated pathway, affect the binding of FBPase to the vacuole was examined. As shown in Fig. 3A, RNase A and glyceraldehyde-3-phosphate dehydrogenase that bind to lysosomal receptor did not affect the binding of FBPase to the vacuole. Other cytosolic proteins, BSA and ovalbumin, also showed no signs of interference for the binding of FBPase to the vacuole. On the other hand, excess amounts of unlabeled FBPase inhibited the FBPase binding to the vacuole almost 80%. The anti-FBPase antibody showed an inhibitory effect on the binding of FBPase to the vacuole (Fig. 3B).

The effect of pH on the binding ability of FBPase was determined. The FBPase binding to the vacuole increased with decreasing pH (Fig. 3C). At the pH values of 5.0 and lower, unlabeled FBPase did not compete for the binding, suggesting the FBPase binding was not specific. The specific binding of FBPase to the vacuole was optimal at pH 5.5-pH 6.0.

Discussion

In this contribution the presence of the FBPase receptor-like protein on the vacuolar membrane has been demonstrated. FBPase was preferentially localized to the vacuole, but not with other organelles in *vid24-1*. FBPase binding to the vacuole was specific and saturable with a Kd of 2.3×10^{-6} M.

Multiple degradation pathways of cytosolic proteins operate within the cells. A selective protein import pathway exists for the uptake and degradation of particular cytosolic proteins by lysosomes. The degradation mechanism of FBPase has not been characterized. Recent reports suggest a vesicle-mediate transport to the vacuole and internalization of vesicles containing FBPase by autophage (Huang and Chiang, 1997; Chiang and Chiang, 1998). However, the possibility of the existence of an alternative pathway for the FBPase degradation cannot be ruled out. Recently the lysosomal membrane glycoprotein LPG96 was identified as a receptor for the selective import and degradation of proteins within lysosomes (Cuervo and Dice, 1996). Also, several lines of evidence suggest that a receptor protein exists that mediates FBPase binding and the uptake into the vacuole (Chiang and Schekman, 1991). Degradation of FBPase requires the synthesis of new proteins and also the early portions of the secretory pathway (Chiang and Schekman, 1991). The requirements for the newly synthesized proteins may involve the synthesis of a FBPase receptor protein that travels the early portions of the secretory pathway *en route* to the vacuole.

Previous efforts in this laboratory to identify the FBPase receptor protein using *pep4* cells have been unsuccessful. This is possibly due to the fact that *pep4* cells contained only a small amount of FBPase that binds to the vacuolar membrane. Because the FBPase uptake is a dynamic process, it is difficult to catch the moment when most of the FBPase are on the vacuolar membrane. In order to improve the chance of identifying the receptor protein, we took advantage of the *vid* mutant, *vid24-1*, that shows FBPase binding to the vacuolar membrane after the re-addition of glucose. *vid24-1* displays interesting phenotypes. FBPase were found on the vacuolar membrane, but failed to be internalized. This suggests a defect in the internalization step in *vid24-1* (Chiang and Chiang, 1998).

Glucose induces a binding of FBPase to the vacuolar membrane, presumably to the receptor, and then the internalization of FBPase would be the subsequent step. The vacuole binding assay developed in this study may provide a useful tool to study processes involved in the receptor recognition. Since FBPase is the only cytosolic protein to be targeted and degraded in the vacuole in a glucose-regulated manner, the binding assay can be used to study whether or not the FBPase receptor is recognized by other cytosolic proteins as well.

To characterize the pathway of FBPase degradation in detail, the purification of the receptor and the cloning of its gene are critical. These studies are in progress in this laboratory. These studies could shed light on the understanding of receptor mediated degradation pathway of cytosolic proteins and organelles by yeast lysosome.

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References

Abeijon, C., Orlean, P., Robbins, P. W. and Hirschberg, C. B. (1989) Topography of glycosylation in yeast: Characterization

of GDP mannose transport and lumenal guanosine diphosphatase activities in Golgi-like vesicles. *Proc. Natl. Acad. Sci.* **86**, 6935-6939.

Chiang, M. C. and Chiang, H. L. (1998) Vid24p, a novel protein localized to the fructose-1, 6-bisphosphatase-containing vesicles, regulates targeting of fructose-1,6-bisphosphatase from the vesicles to the vacuole for degradation. *J. Cell. Biol.* **23**, 1347-1356.

Chiang, H. L. and Schekman, R. (1991) Regulated import and degradation of a cytosolic protein in the yeast vacuole. *Nature* **350**, 313-318.

Chiang, H. L., Terlecky, S. R., Plant, C. P. and Dice, J. F. (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246**, 382-385.

Chvatchko, Y., Howald, I. and Riezman, H. (1986) Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell* **46**, 355-364.

Cuervo, A. M. and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* **273**, 501-503.

Cuervo, A. M., Terlecky, S. R., Dice, J. F. and Knecht, E. (1994) Selective binding and uptake of RNase and glycerate-3-phosphate dehydrogenase by isolated rat liver lysosome. *J. Biol. Chem.* **269**, 26374-26380.

Dice J. (1990) Peptides sequences that target cytosolic proteins for lysosomal proteolysis. *Trends in Biochem. Sci.* **15**, 305-309.

Dunn W. Jr. (1990) Studies on mechanism of autophage: Formation of autophagic vacuole. *J. Cell. Biol.* **110**, 1923-1933.

Finley, D. and Varshavsky, A. (1985) The ubiquitin system: functions and mechanisms. *Trends in Biochem. Sci.* **10**, 343-346.

Hoffman, M. and Chiang, H. L. (1996) Isolation of degradation-deficient mutants defective in the targeting of fructose-1,6-bisphosphatase into the vacuole for degradation in *Saccharomyces cerevisiae*. *Genetics* **143**, 1555-1566.

Huang, P. H. and Chiang, H. L. (1997) Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. *J. Cell. Biol.* **136**, 803-810.

Jentsch S., Seufert, W., Sommer, T. and Reins, H. (1990) Ubiquitin-conjugating enzymes, novel regulators of eukaryotic cells. *Trends in Biol. Sci.* **15**, 195-198.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Munn, A. and Riezman, H. (1994) Endocytosis is required for the growth of vacuolar H-ATPase defective yeast. Identification six new *END* genes. *J. Cell. Biol.* **127**, 373-386.

Raths, S., Rohrer, J., Crausaz, F. and Riezman, H. (1993) *end3* and *end4*: two mutants defective in receptor-mediated and fluid phase endocytosis in *S. cerevisiae*. *J. Cell. Biol.* **120**, 55-65.

Roberts, C. J., Raymond, C. K., Yamashiro, C. T. and Stevens, T. H. (1991) Methods for studying the yeast vacuole. *Methods in Enzymol.* **194**, 644-661.

Rogers, D. T., Hiller, E., Mitsock, L. and Orr, E. (1988) Characterization of the gene for fructose-1,6-bisphosphatase from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Sequence, protein homology, and expression during growth on glucose. *J. Biol. Chem.* **263**, 6051-6057.

Scott, J. H. and Schekman, R. (1980) Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. *J. Bacteriol.* **142**, 414-423.

Seglen, P., Gorden, P. and Holen, I. (1990) Non-selective autophage. *Semin. Cell. Biol.* **1**, 441-448.

Serrano, R. (1983) *In vivo* glucose activation of the yeast plasma membrane ATPase. *FEBS Lett.* **156**, 11-14.

Takeshige, K., Baba, M., Tsuboi, S., Noda, T. and Ohsumi, Y. (1992) Autophage in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell. Biol.* **119**, 301-311.

Terlecky, S. R., Chiang, H. L., Olson, T. S. and Dice, J. F. (1992) Protein and peptide binding and stimulation of *in vitro* lysosomal proteolysis by the 73-kDa heat shock cognate protein. *J. Biol. Chem.* **267**, 9202-9209.

Tsukada, M. and Ohsumi, Y. (1993) Isolation and characterization of autophage-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **333**, 169-174.