Isolation of Degradation-Deficient Mutants Defective in the Targeting of Fructose-1,6-bisphosphatase into the Vacuole for Degradation in *Saccharomyces cerevisiae*

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ABSTRACT

The key regulatory enzyme in the gluconeogenesis pathway, fructose-1,6-bisphosphatase (FBPase), is induced when *Saccharomyces cerevisiae* are grown in medium containing a poor carbon source. FBPase is targeted to the yeast vacuole for degradation when glucose-starved cells are replenished with fresh glucose. To identify genes involved in the FBPase degradation pathway, mutants that failed to degrade FBPase in response to glucose were isolated using a colony-blotting procedure. These vacuolar import and degradation-deficient (*vid*) mutants were placed into 20 complementation groups. They are distinct from the known *sec*, *vps* or *pep* mutants affecting protein secretion, vacuolar sorting and vacuolar proteolysis in that they sort CpY correctly and regulate osmotic pressure normally. Despite the presence of FBPase antigen in these mutants, FBPase is completely inactivated in all *vid* mutants, indicating that the c-AMP-dependent signal transduction pathway and inactivation must function properly in *vid* mutants. *vid* mutants block FBPase degradation by accumulating FBPase in the cytosol and also in small vesicles in the cytoplasm. FBPase may be targeted to small vesicles before uptake by the vacuole.

SEVERAL pathways of protein degradation have been identified in cells (JENSCH *et al.* 1990; SEGLEN *et al.* 1990; CIECHANOVER 1994; HAYNES and DICE 1996). Most short-lived proteins are degraded in the ubiquitin/proteasome pathway (JENSCH *et al.* 1990; CIECHANOVER 1994), while most long-lived proteins are degraded in lysosomes (AHLBERG *et al.* 1985; DICE 1990; SEGLEN *et al.* 1990). Lysosomes can take up protein molecules by a heat shock protein-dependent translocation (CHIANG *et al.* 1989; TERLECKY *et al.* 1992; CUERVO *et al.* 1994), by a nonselective autophagy (KOIMINAMI *et al.* 1983; MORTIMORE *et al.* 1983; HENELL and GLAUMANN 1985; HENELL *et al.* 1987; DUNN 1990a,b; TAKESHIGE *et al.* 1992) or by endocytosis (CHVATCHKO *et al.* 1986; DAVIS *et al.* 1993; RATHS *et al.* 1993; KOLLING and HOLLENBERG 1994; SCHANDEL and JENNESS 1994; VOLLAND *et al.* 1994; LAI *et al.* 1995; RIBALLO *et al.* 1995). Two types of autophagy have been described in mammalian cells (KOIMINAMI *et al.* 1983; MORTIMORE *et al.* 1983; HENELL and GLAUMANN 1985; HENELL *et al.* 1987; DUNN 1990a,b; SEGLEN *et al.* 1990; TAKESHIGE *et al.* 1992). In fed animals, proteins are degraded at basal rates (MORTIMORE *et al.* 1983; HENELL and GLAUMANN 1985; HENELL *et al.* 1987) via microautophagy, which is characterized by an invagination of the lysosomal membrane resulting in pinching off and accumulation of vesicles inside lysosomes (AHLBERG *et al.* 1985; HENELL and GLAUMANN 1985). Cytosolic proteins can be taken up during the invagination process in a nonselective manner (MORTIMORE *et al.* 1983; AHLBERG *et al.* 1985; HENELL and GLAUMANN 1985). When cells are starved of nutrients or serum, protein degradation is induced; it occurs in lysosomes as it can be inhibited by lysosomal inhibitors. The enhanced protein degradation can be achieved through macroautophagy (KOIMINAMI *et al.* 1983; MORTIMORE *et al.* 1983; HENELL and GLAUMANN 1985; DUNN 1990a,b) or a heat shock protein-mediated translocation (CHIANG *et al.* 1989; TERLECKY *et al.* 1992; CUERVO *et al.* 1994). Macroautophagy is characterized by the formation of autophagic vacuoles in the cytoplasm. Autophagic vacuoles are often surrounded by a layer of membrane derived from the endoplasmic reticulum (ER) and mature from AVi (autophage vacuole—initial) to AVd (autophage vacuole—degradative) as they fuse with lysosomes and acquire lysosomal hydrolases (DUNN 1990a,b). Cytosolic proteins can be taken up by macroautophagy in a nonselective fashion (KOIMINAMI *et al.* 1983; HENELL and GLAUMANN 1985). In *Pichia pastoris*, the cytosolic protein, formate dehydrogenase, and peroxisomes are selectively degraded in response to glucose in a *PEP4*-dependent manner (TUTTLE and DUNN 1995).

The key regulatory enzyme in the gluconeogenesis pathway, fructose-1,6-bisphosphatase (FBPase), is induced when the budding yeast *Saccharomyces cerevisiae* is grown in medium containing a poor carbon source (GANCEDO 1971). FBPase is rapidly inactivated when

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glucose-starved cells are replenished with glucose (GANCEDO 1971). Studies using the *pep4* deletion strains have indicated that FBPase is targeted to the vacuole for degradation (CHIANG and SCHEKMAN 1991). The *PEP4* gene encodes the vacuolar proteinase A whose activity is required for the maturation of proteinase B and proteinase C (HASILIK and TANNER 1978; HEMMINGS *et al.* 1981; JONES 1991). Since FBPase targeting into the vacuole is not affected, the import process can be followed in *pep4* cells using cell fractionation or localization techniques. Using indirect immunofluorescence techniques, we have observed that FBPase is localized in the cytosol during growth on acetate and is imported into the vacuole upon transfer of *pep4* cells to glucose (CHIANG and SCHEKMAN 1991). The import of FBPase into the vacuole always occurs whether cells are pregrown on acetate, galactose, oleate or pyruvate to induce FBPase. As long as they are transferred to glucose for 45 min, FBPase is imported into the vacuole in *pep4* cells (CHIANG and SCHEKMAN 1994; CHIANG *et al.* 1996). Using immunoelectron microscopic techniques, the import process has been visualized at the ultrastructural level. FBPase can be found on the vacuolar membrane at the sites of vacuolar invaginations and also with various vesicles inside the vacuole, suggesting that FBPase is taken up by the vacuole by autophagy (CHIANG *et al.* 1996).

The pathway of FBPase degradation seems to be different from the degradation of RNase A that occurs in lysosomes when mammalian cells are starved of serum. The induced degradation of RNase A is dependent on a pentapeptide sequence (KFERQ) and a heat shock protein (CHIANG and DICE 1988; CHIANG *et al.* 1989; TERLECKY *et al.* 1992). A translocation intermediate of RNase A has been reported recently, suggesting that the uptake of RNase A is mediated by a direct translocation of RNase A from the cytosol to lysosomes during serum starvation (TERLECKEY and DICE 1993). Whether FBPase is translocated from the cytosol to the vacuole has not been ruled out completely. However, the staining of FBPase on the vacuolar membrane as well as at the sites where membrane invaginations occur suggests that FBPase uptake is mediated, at least in part, by microautophagy (CHIANG *et al.* 1996).

Upon transfer of cells to glucose, other organelles are also targeted to the vacuole for degradation. Peroxisomes, which are important for the oxidation of fatty acids in *S. cerevisiae,* are induced when cells are grown in oleic acid (ERDMANN *et al.* 1989). Peroxisomes are delivered to the vacuole for degradation when cells are shifted to glucose. Immunoelectron microscopic studies have indicated that peroxisomes are taken up by autophagy. Peroxisomes are engulfed by the vacuole membrane, resulting in the destruction of the whole organelles in the vacuole (TUTTLE and DUNN 1995; CHIANG *et al.* 1996). The glucose-induced degradation of peroxisomes by vacuolar autophagy was observed previously in methyltrophic yeast such as *P. pastoris, H. polymorpha* and *C. boidinii* (BORMANN and SAHM 1978; VEENHUIS *et al.* 1983; TUTTLE and DUNN 1995). In addition to FBPase (CHIANG and SCHEKMAN 1991), formate dehydrogenase (TUTTLE and DUNN 1995) and peroxisomes (BORMANN and SAHM 1978; VEENHUIS *et al.* 1983; TUTTLE and DUNN 1995; CHIANG *et al.* 1996), the plasma membrane proteins such as the maltose transporter (RIBALLO *et al.* 1995) and the galactose transporter (CHIANG *et al.* 1996) are also delivered from the plasma membrane to the vacuole for degradation when cells are transferred to glucose. The degradation of the galactose transporter as well as the maltose transporter is mediated by the endocytic pathway, as mutants defective in the endocytic process block the degradation of these sugar transporters in response to glucose (RIBALLO *et al.* 1995; CHIANG *et al.* 1996).

To identify proteins involved in the pathway of FBPase degradation, we have taken a genetic approach, screening for mutants that fail to degrade FBPase in response to glucose addition. We have identified 20 complementation groups required for the glucose-induced degradation of FBPase. These vacuolar import and degradation mutants (*vid*) are different from the existing *pep, sec* or *vps* mutants affecting protein secretion, vacuolar sorting and vacuolar proteolysis in that they sort CpY and regulate osmolarity normally. They impair the degradation of FBPase, giving half lives ranging from 100 to >300 min, compared to 30 min for wild-type cells. FBPase antigen is found in the cytosol or in punctate structures in the cytoplasm in *vid* mutants. Despite the presence of FBPase antigen in these mutants, FBPase is completely inactivated. We propose that FBPase is first inactivated and then targeted to small vesicles before entering the vacuole.

MATERIALS AND METHODS

Strains, media and antibodies: The strains used in this study were as follows: W303 (*MATα leu2-3,112 ade2 his3-200 trp1-1 ura3-52*) and *pep4* (*MATα leu2-3,112 ade2 his3-200 trp1-1 ura3-52 pep4:TRP1*). *vps8* (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*) was obtained from Dr. S. EMR (University of California, San Diego). YPD contained 1% yeast extract (Difco Laboratories), 2% peptone (Fisher) and 2% glucose. YPKG contained 1% yeast extract, 2% peptone, 1% potassium acetate and 0.5% of glucose. SD (−N) contained 6.7 g/l yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco Laboratories) supplemented with 2% glucose. Nutrients containing 2 μg/ml of adenine, uracil, L-tryptophan and L-histidine and also 3 μg/ml of L-leucine were added to the SD (−N) medium. Antibodies directed against FBPase were generated against purified FBPase. Monoclonal antibodies that recognize the *pro*-sequence of *pre-pro*-CpY and *pro*-CpY were obtained from Dr. T. STEVENS (University of Oregon).

Isolation of *vid* mutants: Yeast strain W303 was UV mutagenized to 30% survival, plated on YPKG plates, incubated for 4 days at room temperature to induce FBPase, and then

transferred to nitrocellulose membranes. Cells were immersed in 2 ml of YP containing 5% glucose for 2 hr at 37°, lysed with 2 ml of lysis buffer containing 0.1% SDS, 0.2 M NaOH, 35 mM dithiotreitol for 30 min and washed with Tris-buffered saline (TBS) solution containing 0.5 M Tris and 1.5 M NaCl pH 7.4. Nitrocellulose membranes containing cellular lysates were subsequently incubated with 10 ml of 2% milk in TBS-T (TBS with 0.1% Tween-20) for 30 min and 10 ml of 1:500 dilution of affinity-purified anti-FBPase antibodies in TBS-T with 2% milk for another hour. After several washes with TBS-T, the membranes were incubated with 10 ml of TBS-T containing 1:1000 dilution of Donkey anti-rabbit antibodies conjugated with horse-radish peroxidase (Amersham) for 1 hr at room temperature. Membranes were washed three times with TBS-T for 15 min, rapidly rinsed with 2 ml ECL reagents (Du Pont) and exposed on X-ray films (Kodak) for 20 sec.

Immunoblotting: Yeast cells were grown in YPKG for 48 hr at 30° and transferred to YPD at 37° for 75 min. Cell were lysed by adding equal volume of glass beads and agitating vigorously. Samples were boiled at 95° for 5 min. Proteins were separated on 10% SDS gels, transferred to a nitrocellulose membrane and blotted with 1:1000 dilution of affinity-purified anti-FBPase antibodies followed by 1:10,000 dilution of HRP-conjugated anti-rabbit antibodies and exposed on X-ray films after ECL reaction. To separate P1 and P2 form of *pro*-CpY from mature CpY, proteins were separated on 7% SDS gels and anti-CpY antibodies were used at 1:1000 dilution.

Complementation analysis: Standard yeast genetic techniques were used for complementation and tetrads dissection (GUTHRIE and FINK 1991). Mutants were backcrossed to W303 four to five times. Segregation of *vid* mutations was examined by immunoblotting following the shift up regimen.

CpY sorting: Sorting of CpY was studied using wild type, *pep4* and *vps8* as controls (ROBINSON *et al.* 1988). Cells were spheroplasted with zymolyase-100 T (ICN Biomedicals, Inc.) with a final activity of 30 U per optical density unit at 600 nm. Spheroplasts were labeled with Tran^{35}S label (0.2 mCi/ml) (Du Pont, specific activity = 10 mCi/ml) for 20 min at 30° and chased in medium containing excess unlabeled methionine and cysteine for 30 min at 30°. The labeled culture was separated into spheroplasts (intracellular) and medium (extracellular) fractions and immunoprecipitated with antiserum to CpY. The plate test of LATTERICH and WATSON (1991) for osmotic regulation was performed using *ssv* mutants as controls.

Degradation of FBPase: Cells were precultured overnight and then labeled in 10 ml yeast nitrogen base without sulfate and amino acids containing 2% oleic acid and 300 μCi of ^{35}S-methionine and cysteine (Du Pont, specific activity = 10 mCi/ml) for 24 hr. Cells (2 ml) were shifted to SD (−N) containing 2% glucose, supplements and excess unlabeled L- methionine and L-cysteine (20 μg/ml) for 0, 45, 90, 180 or 300 min. Cell extracts were obtained and immunoprecipitated with 5 ml of anti-FBPase, followed by protein A-Sepharose (Pharmacia). Proteins were separated on 10% SDS gels, dried, exposed in phosphoimager cassettes, and quantified with a phosphoimager (Molecular Dynamics). Degradation of FBPase was determined using t = 0 as 100%. The half life of FBPase degradation is defined as the time required for 50% of FBPase to be degraded after glucose readdition.

Degradation of long-lived proteins: Cells (10 ml) were grown and labeled for 24 hr as described above. Cells (2 ml) were transferred to SD (−N) containing 2% glucose and supplements with excess unlabeled L-methionine and L-cysteine (20 μg/ml) for 0, 45, 90, 180 or 300 min. Cells were centrifuged and the supernatants (2 ml) were precipitated with 10% trichloroacetic acid (TCA) with 50 ml of 2% bovine serum albumin at 4°. Samples were counted with a liquid scintillation counter. Total radioactivity was determined by measuring cell-associated counts plus counts in the medium. Degradation of long-lived proteins was calculated as the percentage release of radioactivity into a TCA-soluble fraction in the medium. The release of methionine and cysteine to the medium may also come from the vacuolar pool. In the absence of glucose, both wild-type and *pep4* cells show minimal rates of TCA soluble activity (<0.01%/hr). Upon glucose readdition, the TCA-soluble activity in the medium is induced to 5%/hr in wild-type cells. Since *pep4* cells show a rate of 1.3%/hr (CHIANG *et al.* 1996), the difference between wild-type and *pep4* cells (3.7%/hr) may reflect the glucose-induced release of methionine and cysteine to the medium due to vacuolar proteolysis.

TABLE 1

Scheme for the isolation of *vid* mutants

No. of colonies after UV mutagenesis	30,000
No. of mutants after first screening with colony blotting procedure	369
No. of mutants accumulating precursors of CpY as detected by colony lifting assay	75
No. of mutants further screened by Western blotting of FBPase	40
No. of mutants with half lives of FBPase degradation longer than 100 min	29
No. of mutants missorting CpY to the cell surface	0
No. of alleles in each complementation group	
vid1–11	1
vid12	2
vid13	4
vid14–17	1
vid18	2
vid19	3
vid20	3
Final complementation groups	20

Immunofluorescence microscopy: Indirect immunofluorescence microscopy was performed as described (CHIANG and SCHEKMAN 1991).

FBPase enzyme activity assay: Cells (OD_{600} = 20) were lysed by adding 200 μg of glass bead and agitating vigorously. Cellular lysates were used to assess FBPase activity by the formation of NADPH at 340 nm (CHIANG and SCHEKMAN 1991).

Cell fractionation: Yeast cells were grown in 500 ml of YPKG for 2 days at 30° in an environmental shaker (300 rpm). Cells were harvested by centrifugation at 500 × *g* for 5 min and divided. Half of the cells (250 ml) were shifted to YPD for 60 min at 30°. At the end of incubation, 10 mM NaN_3 was added to the culture medium and yeast cells were collected by centrifugation. Cells were resuspended in 50 ml of 100 mM Tris-HCl pH 9.5, 100 mM β-mercaptoethanol, 10 mM NaN_3 and 40 mM EDTA and incubated at 30° for 45 min with gentle shaking (50 rpm). Cells were harvested and spheroplasts prepared by incubating cells in 50 ml of 1.5 M sorbitol, 10 mM Tris-HCl pH 7.5 with 10 units of zymolyase-100T (ICN Biomedicals, Inc.) per $O.D_{600}$ unit of cells at 30° for 30 min. Spheroplasts were collected by centrifugation and washed once with 10 ml of 1 M sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES pH 6.8 and resuspended in 10 ml TEA buffer (10 mM triethanolamine pH 7.5, 100 mg/ml phenylmethyl sulfonyl fluoride, 10 mM NaF, 10 mM NaN_3, 1 mM EDTA and 0.8 M sorbitol). Spheroplasts were

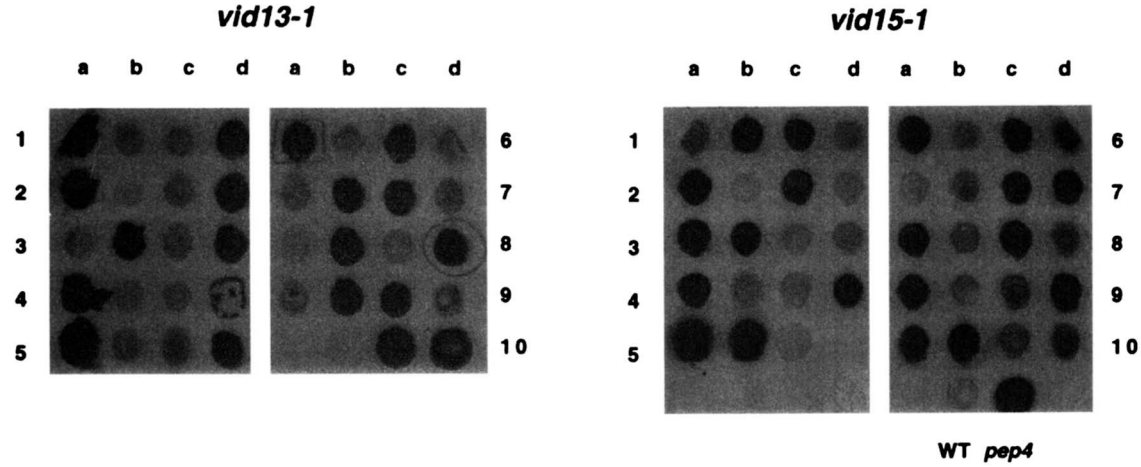


FIGURE 1.—Colony blotting of FBPase degradation in tetrads derived from the *vid13-1* and *vid15-1* mutants. FBPase degradation mutants were outcrossed to wild-type cells. Tetrads were dissected and grown on YPKG plates to induced FBPase. Cells were transferred to nitrocellulose membranes and shifted to YPD (5%) glucose as described in MATERIALS AND METHODS. Mutants were identified as dark circles after ECL reactions. Tetrads are numbered as a, b, c, and d. Representative colony blotting of sets of 10 tetrads (#1–10) are shown for the *vid13-1* (left) and the *vid15-1* mutants (right). Wild-type and *pep4* cells were shown on the lowest part of the right panel.

homogenized using a Dounce homogenizer with 20 strokes on ice. Cell lysates were centrifuged first at 10,000 × *g* for 20 min. The supernatant was further centrifuged at 100,000 × *g* for 2 hr to obtain the high speed supernatant (S) and pellet (P). To estimate whether FBPase sedimented in the high speed pellet after glucose readdition, 5000-fold dilution of proteins from the total (T), supernatant (S) and pellet (P) were loaded on SDS-PAGE gels. The distribution of FBPase and Pma1p were determined by Western blotting. The pellet (P) was resuspended in 1 ml of TEA buffer, loaded on a 90 × 1.5 cm Sephacryl S-1000 (Pharmacia) column and eluted with 200 ml of TEA buffer with a flow rate of 8 ml/hr. Fractions (4 ml) were collected and 1.5 ml of samples were precipitated with TCA at the final concentration of 10% at 4° for 1 hr on ice. Samples were centrifuged at 13,000 × *g* for 10 min at 4° and pellets washed once with 1 ml ice-cold acetone and resuspended in 50 μl of SDS sample buffer. Proteins (15 μl) were separated on 10% SDS-PAGE gels and immunoblotted with anti-FBPase antibodies. We initially collected 50 samples and found that most proteins such as Pma1p, FBPase and hemoglobin (soluble protein marker) fractionated between #10 and 30. The positions of the plasma membrane ATPase (Pma1p) were determined by anti-Pma1p antiserum. Hemoglobin fractionated at #30 on the Sephacryl S-1000 chromatography.

RESULTS

Isolation of *vid* mutants: Mutants defective in the glucose-induced degradation of FBPase were screened with anti-FBPase antibodies using a colony blotting procedure. Cells were UV mutagenized and grown for 4 days on YPKG plates containing acetate to induce FBPase; colonies were transferred to nitrocellulose membranes and immersed in YPD for 2 hr at 37°. During this regimen, wild-type cells degrade FBPase completely. Of 30,000 colonies from mutagenized cells, 369 were reactive with anti-FBPase antibody and apparently failed to degrade FBPase (Table 1). They were further

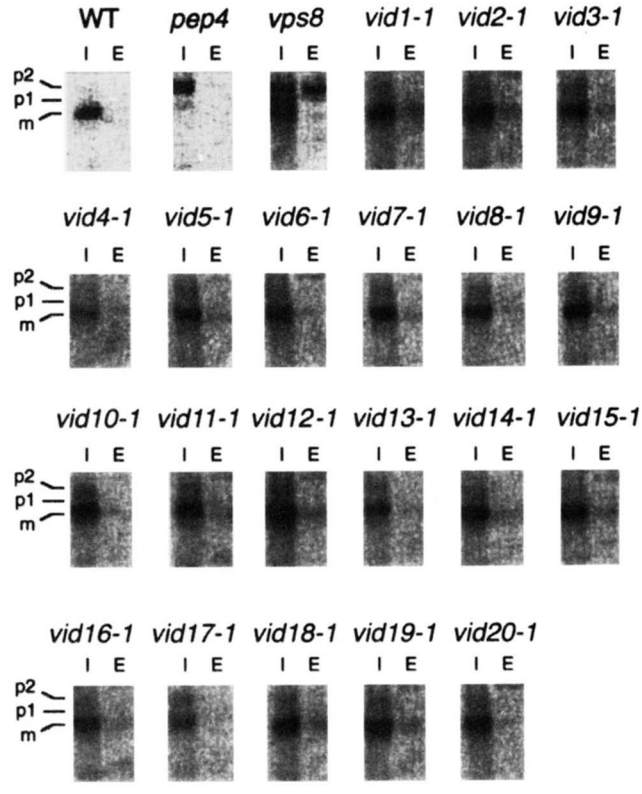


FIGURE 2.—CpY is sorted normally in *vid* mutants. Wild type, *pep4*, *vps8* and *vid1–vid20* were spheroplasted, radiolabeled and chased as described in MATERIALS AND METHODS. Proteins from the intracellular and extracellular fractions were precipitated with anti-CpY antibodies followed by Protein A Sepharose. Proteins were separated on 7% SDS-PAGE gels and exposed using a phosphoimager cassette. I, intracellular fractions; E, extracellular fractions; p1, the ER (67 kDa) form of CpY; p2, the Golgi (69 kDa) form of CpY; m, the mature form of CpY (61 kDa).

TABLE 2

Class A *vid* mutants

VID genotype	$t_{1/2}$ of FBPase degradation (min)	Localization of FBPase	$t_{1/2}$ of FBPase inactivation (min)	Degradation of long-lived proteins (%/hr)[a]
VID	30	Nondetectable	20	5 (100)
vid1-1	300	Cytosol	20	5 (100)
vid2-1	125	Cytosol	20	5 (100)
vid3-1	150	Cytosol	20	5 (100)
vid4-1	>300	Cytosol	20	5 (100)
vid5-1	100	Cytosol	20	5 (100)
vid6-1	115	Cytosol	20	5 (100)
vid7-1	240	Cytosol	20	5 (100)
vid8-1	110	Cytosol	20	5 (100)
vid9-1	120	Cytosol	20	5 (100)
vid10-1	135	Cytosol	20	5 (100)
vid11-1	110	Cytosol	20	5 (100)
vid12-1,2	180	Cytosol	20	5 (100)
vid13-1,2,3,4	>300	Cytosol	20	5 (100)
vid14-1	110	Vesicles	20	5 (100)
vid15-1	200	Vesicles	20	5 (100)
vid16-1	200	Vesicles	20	5 (100)

[a] Values in parentheses are.

screened for the presence of *pep, sec* and *vps* mutations, since we showed previously that FBPase degradation is delayed in the *pep4* mutant, which is defective in vacuolar proteinases (HASILIK and TANNER 1978; HEMMINGS *et al.* 1981; JONES 1991) and also in *sec* mutants blocked in the early portions of the secretory pathway (CHIANG and SCHEKMAN 1991), and since FBPase is targeted to the vacuole for degradation. All three classes of mutants, *pep,* early *sec,* and *vps* accumulate ER (p1) or Golgi-modified (p2) precursors of carboxypeptidase Y (CpY), both of which retain the *pro*-peptide of the precursor (HASILIK and TANNER 1978; HEMMINGS *et al.* 1981; ROTHMAN and STEVENS 1986; ROBINSON *et al.* 1988; JONES 1991) and were identified by a colony lift assay employing monoclonal antibodies that specifically recognize the *pro*-sequence of the CpY precursors. A total of 75 such strains were identified and eliminated. The remaining 294 strains were examined for FBPase degradation defects using Western blotting with affinity-purified anti-FBPase antibodies; 40 *vid* mutants with strong degradation defects were chosen for further study. The remaining 254 mutants showed weak defects in FBPase degradation.

Upon transfer of cells to glucose from a medium containing a poor carbon source, the preexisting FBPase is inactivated and degraded. The transcription of FBPase is also downregulated so that no new FBPase is synthesized (SEDIVY and FRAENKEL 1985). Immunoblotting detects the steady-state level of FBPase and does not distinguish between defects in degradation of FBPase or the downregulation of transcription. To distinguish, we determined the kinetics of FBPase degradation in the remaining 40 mutants. In wild-type cells, FBPase is degraded with a half life of 30 min in response to glucose. Cells that showed half lives of FBPase degradation <100 min were considered to be weak mutants; 11 such strains were identified. The remaining 29 *vid* mutants gave FBPase half lives from 100 min to >300

TABLE 3

Class B *vid* mutants

VID genotype	$t_{1/2}$ of FBPase degradation (min)	Localization of FBPase	$t_{1/2}$ of FBPase inactivation (min)	Degradation of long-lived proteins (%/hr)[a]
VID$^+$	30	Nondetectable	20	5.0 (100)
vid17-1	150	Cytosol	20	2.5 (50)
vid18-1,2	200	Cytosol	20	2.5 (50)
vid19-1,2,3	150	Cytosol	20	3.0 (60)
vid20-1,2,3	>300	Cytosol	20	3.0 (60)

[a] Values in parentheses are.

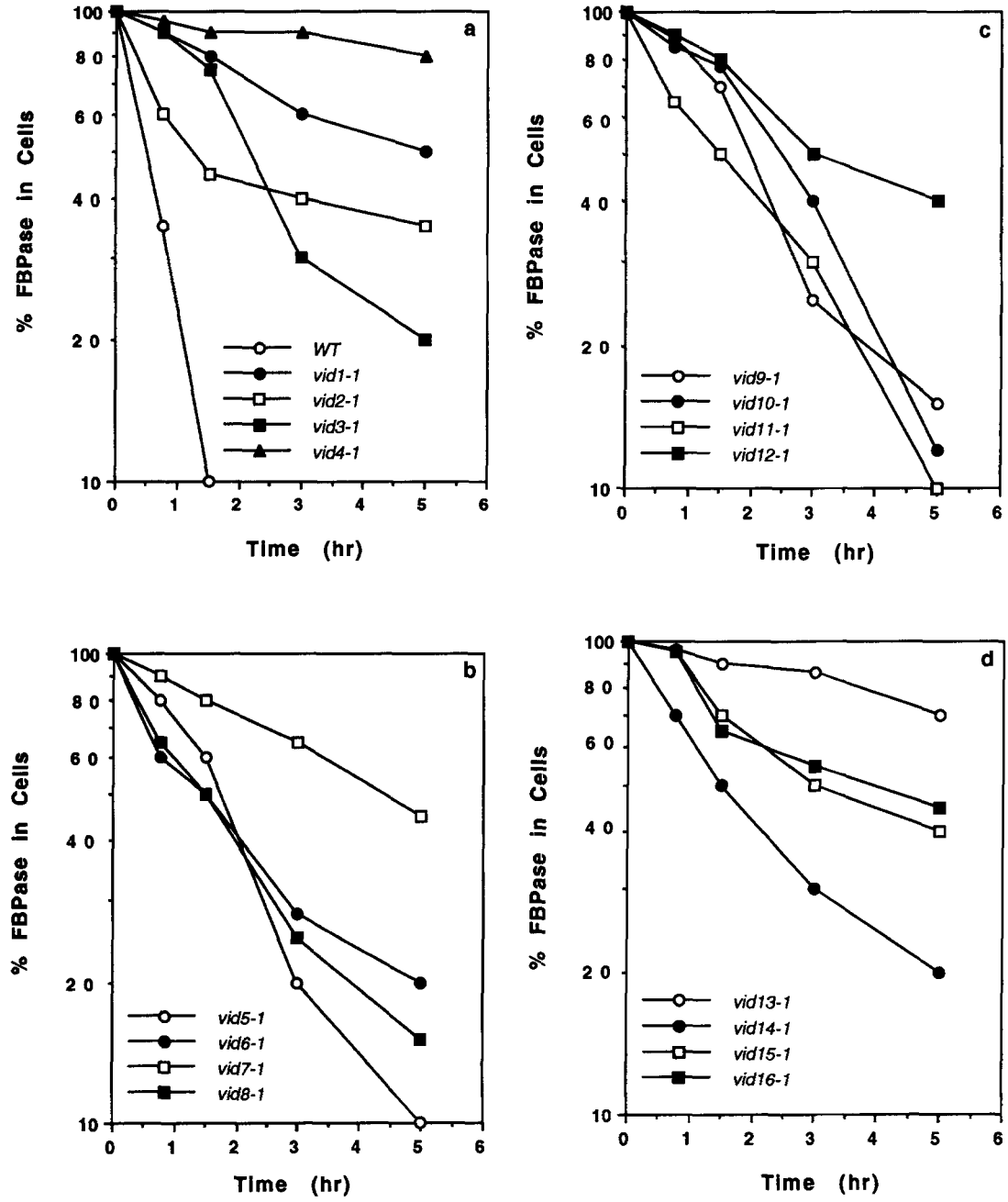


FIGURE 3.—Class A mutants are impaired in the degradation of FBPase. Wild type and *vid1–vid16* were radiolabeled and chased in SD (−N) medium containing 2% glucose for 0, 45, 90, 180 or 300 min. Cell-free extracts were obtained, immunoprecipitated with anti-FBPase antibodies, separated on 10% SDS-PAGE gels and exposed using a phosphoimager cassette. The kinetics of FBPase degradation were determined as described in MATERIALS AND METHODS. The degradation of FBPase was shown in wild type, *vid1–vid4* (a), *vid5–vid8* (b), *vid9–vid12* (c) and *vid13–vid16* (d). The degradation of long-lived proteins was performed as described in MATERIALS AND METHODS. Representative results of the degradation of long-lived proteins are shown in wild type, *vid1-1*, *vid2-1*, *vid9-1*, *vid14-1* and *vid16-1* (e).

min. These 29 mutants were outcrossed to a wild-type strain four to five times and the segregation of FBPase degradation defects in tetrads followed by the colony blotting method. Representative results of 10 tetrads derived from the *vid13-1* and *vid15-1* mutants are shown in Figure 1. Patches of FBPase degradation-defective mutants reacted with anti-FBPase antibodies and appeared as dark circles. Of the 20 tetrads shown in Figure 1, one segregant from the *vid15-1* cross (#6d) showed a partial FBPase degradation defect. When this clone was tested for FBPase degradation using Western blotting, it was wild type for FBPase degradation. Therefore, FBPase degradation-defective mutants were first screened by the colony blotting procedure, further ana-

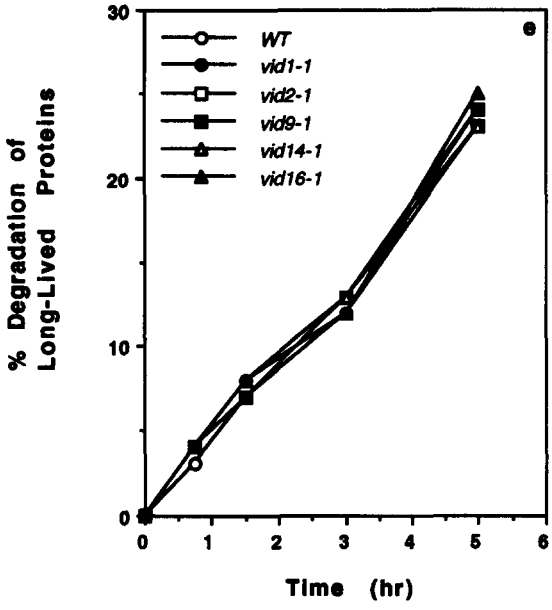
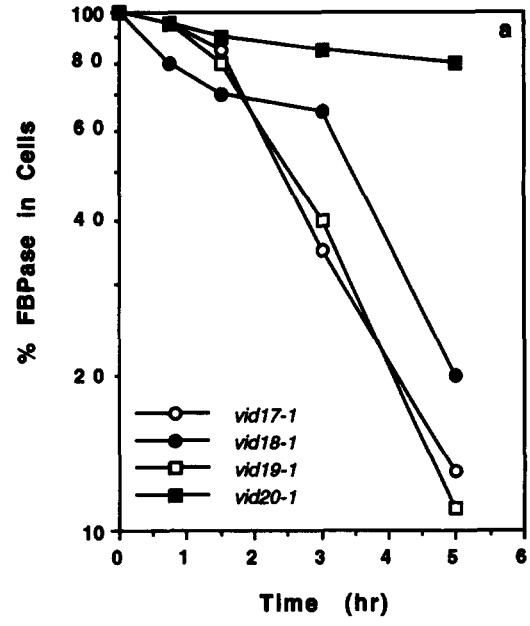


FIGURE 3.—*Continued*



lyzed by Western blotting and finally confirmed by pulse-chase experiments.

Since all *vid* heterozygotes were wild type for FBPase degradation, all 29 mutations were recessive. The mutants were crossed in all possible combination to test for complementation. A total of 20 complementation groups were identified. Table 1 lists the distribution of alleles within complementation groups. Single alleles were found for *vid1–vid11* and also for *vid14–vid17,* two alleles for *vid12* and *vid18,* three alleles for *vid19* and *vid20,* and four alleles for *vid13.*

Sorting of CpY is not significantly altered in *vid* mutants: The sorting of CpY was studied using three methods in addition to the colony blot using anti *pro*CpY monoclonal antibodies. The 40 putative *vid* mutants were examined for the accumulation of the precursor forms of CpY in cell-free extracts after SDS-PAGE electrophoresis using antibodies to mature CpY. All 40 *vid* mutants tested contained the 61-kD, mature form of CpY in total cell extracts. Kinetic studies of CpY maturation and sorting were performed on spheroplasts of the 29 strains that displayed half lives of FBPase degradation longer than 100 min. Figure 2 shows the immunoprecipitation of CpY in the intracellular (I) and extracellular (E) fractions from wild-type, *pep4, vps8* and *vid1–vid20* mutants after a 30 min chase. The wild type contains mature CpY within the cell; the *pep4* mutant contains the P2 precursor intracellularly. The *vps8* mutant, which missorts CpY (ROTHMAN *et al.* 1988), contains p1, p2 and mature CpY intracellularly but also secretes precursors. Mutants representative of the 20 *vid* complementation groups all showed normal maturation of CpY as demonstrated by the presence of the mature form of CpY in the intracellular fractions. When

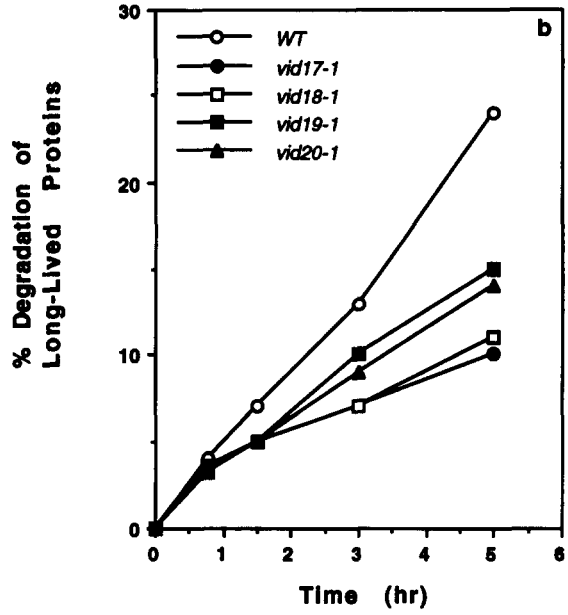


FIGURE 4.—Class B mutants have additional defects in degrading long-lived proteins. Pulse-chase and immunoprecipitation of FBPase were performed as described. The kinetics of FBPase degradation are shown for *vid17-1, vid18-1, vid19-1* and *vid20-1* (a). The rates degradation of long-lived proteins are shown for wild-type cells, *vid17-1, vid18-1, vid19-1* and *vid20-1* (b).

the results were quantitated, <5% of the CpY forms were extracellular. Therefore, the sorting of CpY is not significantly altered in *vid* mutants. A trace amount of p2 was found in some mutants, suggesting that CpY processing may be slowed in these mutants.

Finally, all mutants were able to grow on YPD plates containing 2 M NaCl, 2.5 M glycerol or 2.5 M sorbitol,

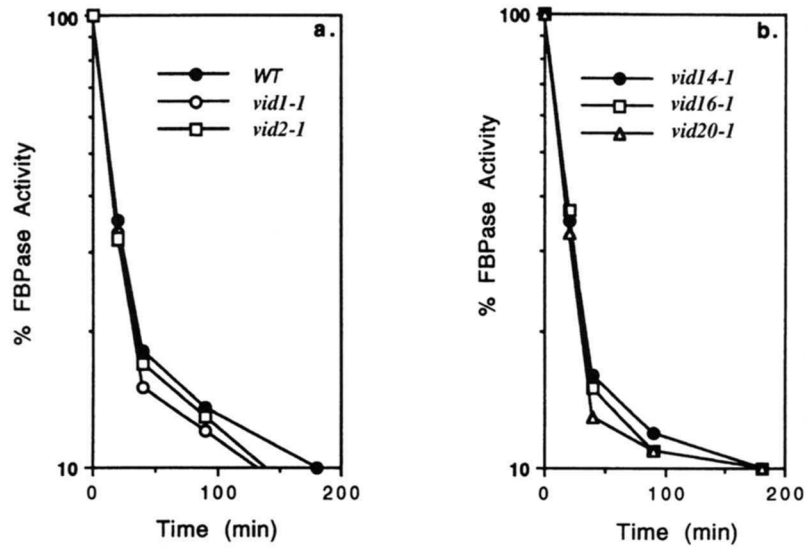


FIGURE 5.—FBPase is inactivated in response to glucose. Yeast cells were grown in YPKG to induce FBPase and shifted to YPD at timed intervals. Representative results of the kinetics of FBPase inactivation are shown for wild-type cells, *vid1-1* and *vid2-1* mutants (a), *vid14-1, vid16-1* and *vid20-1* mutants (b).

indicating that osmoregulation was normal. Since the vacuole plays an important role in osmoregulation (LATTERICH and WATSON 1991), vacuole function appears normal.

Class A *vid* mutants impair the degradation of FBPase: The mutants defective in the glucose-induced degradation of FBPase fell into 20 different complementation groups (Table 1). To further subdivide the mutants, we examined the degradation of long-lived proteins, since we showed previously that glucose induces a massive degradation of long-lived proteins at a rate of 5%/hr. This glucose-induced protein degradation occurs primarily in the vacuole, as *pep4* mutants block the induced protein degradation in response to glucose (CHIANG *et al.* 1996). We have found that representatives of 16 complementation groups (*vid1–vid16*) degrade long-lived proteins at a normal rate (Table 2). We call these Class A mutants. Representatives of four complementation groups (*vid17–vid20*) are defective in degrading long-lived proteins as well as FBPase (Table 3). We call these class B mutants.

Figure 3 shows the kinetics of FBPase degradation in

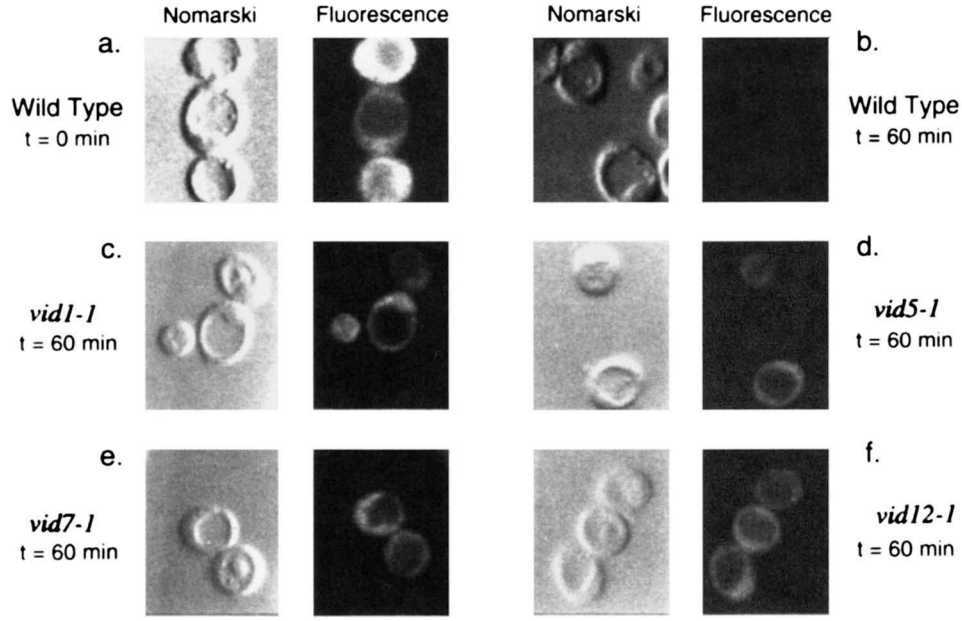


FIGURE 6.—Localization of FBPase in *vid* mutants. Indirect immunofluorescence microscopy of FBPase staining at t = 0 min and at t = 60 min in wild-type cells (a and b), and also in *vid1-1* (c), *vid5-1* (d), *vid7-1* (e) and *vid12-1* (f) mutants that showed a cytosolic staining of FBPase at t = 60 min.

Class A mutants. In wild-type cells, FBPase is degraded with a half life of 30 min and <10% of the FBPase can be detected after a chase in glucose for 90 min. Class A mutants degrade FBPase with half lives ranging from 100 to >300 min and the kinetics of FBPase degradation differ from one mutant to another. For example, *vid1-1* mutants degrade FBPase with a half life of 300 min; 50% of the FBPase remains after 5 hr of chase in glucose. *vid2-1* mutants degrade FBPase with a half life of 125 min; 35% of the FBPase remains after 5 hr of chase in glucose. *vid1-1, vid4-1* and *vid13-1, 2, 3, 4* mutants degrade FBPase with half lives of 300 min or more and are considered to be strong mutants. *vid7-1, vid15-1* and *vid16-1* mutants show half lives of FBPase degradation ranging from 200 to 300 min and are considered to have intermediate degradation defects. Most mutants have half lives of FBPase degradation varying from 100 to 200 min. These mutants, *vid2-1, vid3-1, vid5-1, vid6-1, vid8-1, vid9-1, vid10-1, vid11-1, vid12-1, 2* and *vid14-1,* are weaker mutants. Class A mutants degrade long-lived proteins at a rate similar to that of wild-type cells (Table 2). Representative results of the degradation of long-lived proteins are shown for *vid1-1, vid2-1, vid9-1, vid14-1* and *vid16-1* (Figure 3e).

Class B mutants are defective in degrading FBPase and long-lived proteins: We have identified four Class B mutants that are defective in degrading both FBPase and long-lived proteins (Table 3). Figure 4a shows that the *vid17-1, vid18-1, vid19-1* and *vid20-1* mutants degraded FBPase with half lives of 150, 200, 150 and >300 min, respectively. The degradation of long-lived proteins was reduced to 50% of the wild-type level in *vid17-1* and *vid18-1* and to 60% in *vid19-1* and *vid20-1.* (Figure 4b). All Class B *vid* mutants accumulate FBPase in the cytosol (Table 3). Since Class B mutants have an additional defect in degrading long-lived proteins, they may affect more global aspects of the degradation process, affecting the degradation of FBPase and other cytosolic proteins.

FBPase is inactivated in all *vid* mutants: If FBPase remained active during fermentative growth, it would generate a futile cycle of simultaneous gluconeogenesis and glycolysis, resulting in wasting of ATP. Failure to degrade FBPase might be detrimental to the growth of *vid* mutants if FBPase remained active. All *vid* mutants grew normally, with a doubling time of 90–110 min (data not shown), suggesting that FBPase might not function in *vid* mutants. This possibility was examined by measuring the activity of FBPase at several time intervals after glucose readdition. As was reported by GANCEDO (1971), in wild-type cells, FBPase was inactivated rapidly with 50% of FBPase being inactivated within 20 min after a shift to glucose. We found that all *vid* mutants inactivated FBPase at rates similar to that of wild-type cells. Figure 5 shows representative results of FBPase inactivation for the *vid1-1, vid2-1, vid14-1, vid16-1* and *vid20-1* mutants. Since FBPase was completely inactivated, no energy futile cycle was produced in *vid* mutants.

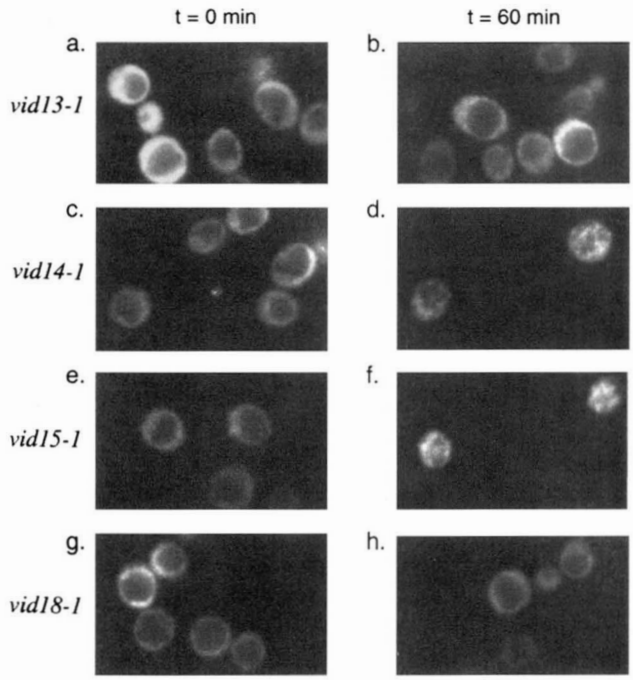


FIGURE 7.—Distribution of FBPase in *vid13-1, vid14-1, vid15-1* and *vid18-1* mutants. Indirect immunofluorescence microscopic studies of FBPase that show FBPase staining at t = 0 min and at t = 60 min in the *vid13-1* (a and b), *vid14-1* (c and d), *vid15-1* (e and f) and *vid18-1* (g and h).

FBPase is found in the cytosol and also in punctate structures in *vid* mutants: To determine the site of blockage along the FBPase degradation pathway in *vid* mutants, we studied the subcellular localization of FBPase using indirect immunofluorescence microscopy. Cells were glucose-starved, transferred to glucose for 60 min, fixed and processed for immunofluorescence microscopic studies. As shown in Figure 6, wild-type cells accumulated FBPase in the cytosol at t = 0 min (Figure 6a). Upon refeeding of cells with glucose for 60 min, no FBPase was detected, indicating a complete degradation of FBPase in wild-type cells (Figure 6b). All *vid* mutants accumulated FBPase in the cytosol at t = 0 min. However, FBPase was not degraded in these mutants. Immunofluorescence studies indicated that most of the *vid* mutants accumulate FBPase in the cytosol after glucose readdition for 60 min (Table 2, Table 3 and Figure 6). Representative results of cytosolic staining of FBPase at t = 60 min are shown in *vid1-1* (Figure 6c), *vid5-1* (Figure 6d), *vid7-1* (Figure 6e) and *vid12-1* (Figure 6f).

Figure 7 shows the time-dependent distribution of FBPase in the *vid13-1, vid14-1 vid15-1* and *vid18-1.* At t = 0 min, FBPase staining was in the cytosol (Figure 7a). FBPase remained in the cytosol in *vid13-1* at t = 60 min (Figure 7b). Different patterns of FBPase staining were observed in the *vid14-1, vid15-1* and *vid16-1* mutants.

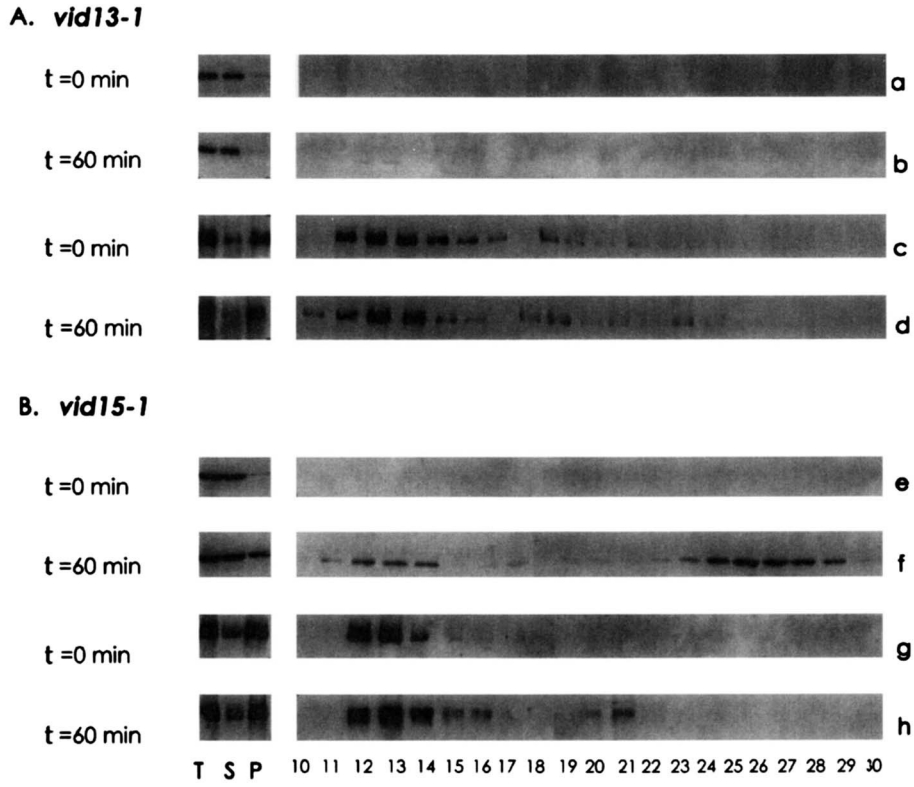


FIGURE 8.—Cell fractionation of FBPase in the *vid13-1* and *vid15-1* mutants. *vid13-1* and *vid15-1* were grown in YPKG medium and shifted to glucose. Spheroplasts were prepared and fractionated as described in MATERIALS AND METHODS. The distribution of FBPase and Pma1p in the total (T), supernatant (S) and pellet (P) were determined by immunoblotting with anti-FBPase and anti-Pma1p antibodies. The pellets were further separated on a Sephacryl S-1000 chromatography. Fractions are shown from #10–30. Pma1p fractionates at #12–14, the soluble proteins using hemoglobin as a marker fractionates at #30. A, *vid13-1* at t = 0 min and t = 60 min; a and b, FBPase; c and d, Pma1p; B, *vid15-1* at t = 0 min and t = 60 min; e and f, FBPase; g and h, Pma1p.

At t = 0 min, FBPase was localized in the cytosol in *vid14-1* (Figure 7c), but 60 min after transfer to glucose, FBPase staining was now localized in punctate structures in the cytoplasm in the *vid14-1* mutant (Figure 7d). Similarly, in the *vid15-1* mutant, FBPase was localized in the cytosol at t = 0 min (Figure 7e) but was found in punctate structures in the cytoplasm 60 min after transfer to glucose (Figure 7f). *vid17–vid20* have additional defects in degrading long-lived proteins. They all block FBPase degradation by accumulating FBPase in the cytosol (Table 3). Representative results of cytosolic staining of FBPase at t = 0 min (Figure 7g) and t = 60 min are shown for the *vid18-1* mutant (Figure 7h).

Cell fractionation of FBPase: Punctate staining of FBPase in the *vid14-1, vid15-1* and *vid16-1* mutants suggested that FBPase was associated with subcellular structures in these mutants. If this were the case, FBPase might be sedimented in the high speed pellet. The distribution of FBPase in the supernatant (S) or pellet (P), before and after glucose shift in the *vid13-1* and *vid15-1* mutants were examined by Western blotting. To separate intracellular organelles, the pellets were further fractionated on a Sephacryl S-1000 chromatography. The *vid13-1* mutant, which accumulated FBPase in the cytosol, was used as a control. Most of the FBPase was present in the supernatant at either t = 0 or t = 60 min. On the S-1000 chromatography, no FBPase was detected (Figure 8, a and b). We used the plasma membrane ATPase (Pma1p) as a control and found that Pma1p was present mostly in the pellet. It fractionated at positions 12–14 both at t = 0 min and t = 60 min (Figure 8, c and d). *vid15-1* showed FBPase staining in punctate structures after glucose shift (Figure 7f). At t = 0 min, FBPase was present in the supernatant (Figure 8e). After glucose readdition for 60 min, ~30–40% of the total FBPase was sedimentable in the pellet. On the S-1000 chromatography, FBPase was detected in two peaks: one at fractions 12–14, another at 24–28 (Figure 8f). In the *vid15-1* mutant, Pma1p partitioned mostly in the pellet. It fractionated at positions 12–14; its distribution was not affected by glucose in either the *vid15-1* or the *vid13-1* mutants (Figure 8, g and f).

DISCUSSION

Using a colony blotting procedure, we have identified 20 mutants defective in the glucose-triggered degrada-

tion of FBPase. These mutants are different from the existing *pep, sec* or *vps* mutants, because they process and sort CpY normally and show normal osmoregulation. Based on these criteria, they are unlikely to be known mutants that affect the secretory pathway, the sorting of proteins to the vacuole or proteolysis in the vacuole. *vid* mutants are also different from the existing mutants that impair the c-AMP-dependent signal transduction pathway (LAMPONI *et al.* 1987; TOYODA *et al.* 1987), for FBPase is completely inactivated in *vid* mutants.

Inactivation of FBPase precedes the degradation of the protein: An interesting feature of *vid* mutants is that no significant growth defect is observed in *vid* mutants during fermentative growth. FBPase activity decreases rapidly following a transfer of *vid* mutants to glucose. The inactivation occurs at a rate similar to that of wild-type cells in spite of the presence of FBPase protein in *vid* mutants. Therefore, no futile cycling of gluconeogenesis and glycolysis occurs in *vid* mutants. In wild-type cells, the inactivation of the FBPase enzyme and the degradation of the FBPase protein occur so rapidly that these two processes cannot be separated. Using *vid* mutants, the inactivation can now be separated from the degradation process. Thus, catabolite inactivation of FBPase occurs in two sequential steps: inactivation of the enzyme followed by the degradation of the protein in the vacuole.

FBPase is associated with subcellular structures in the *vid14*, *vid15* and *vid16* mutants: Immunolocalization experiments have revealed two patterns of FBPase staining in *vid* mutants: in some mutants, FBPase can be found in the cytosol; in others it is found in punctate structures in the cytoplasm. The punctate staining of FBPase in the *vid14–vid16* mutants suggests that FBPase is associated with small vesicles in the cytoplasm. This was further supported by cell fractionation, for a substantial amount of FBPase was recovered in the 100,000 g pellet after transfer of the *vid15-1* mutant to glucose for 60 min. On Sephacryl S-1000 chromatography, FBPase appears as two distinct peaks in the *vid15-1* mutant, one at fractions #12–14, another at #24–28. The first peak overlaps with Pma1p, FBPase may be associated with subcellular structures with similar size to the fragmented or recircularized plasma membrane. A greater amount of FBPase is found in the second peak, which may represent a different type of structure. If FBPase targeting to the vacuole occurs in a sequential manner such that FBPase is inactivated first in the cytosol, targeted to small vesicles and then to the vacuole, the mutations causing accumulation of FBPase in the cytosol (*vid1–vid13*) may affect early steps of the FBPase degradation pathway, whereas the *vid14–vid16* mutations, which result in vesicle-associated FBPase, may interfere with the FBPase degradation pathway at later stages by blocking the fusion of small vesicles with the vacuole. The *vid17–vid20* mutants are defective in degrading long-lived proteins as well as FBPase. Cloning of *VID* genes may provide insights into the understanding of the molecular mechanisms responsible for the targeting of FBPase into the vacuole for degradation.

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