Proteins of Newly Isolated Mutants and the Amino-terminal Proline Are Essential for Ubiquitin-Proteasome-catalyzed Catabolite Degradation of Fructose-1,6-bisphosphatase of Saccharomyces cerevisiae*

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Marcus Hämmerle†‡§, Jürgen Bauer‡¶§, Matthias Rose¶, Alexander Szallies‡, Michael Thumm‡, Stefanie Düsterhus, Dieter Mecke**, Karl-Dieter Entian†‡‡, and Dieter H. Wolf‡†§§

From the †Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany, the ‡Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität Frankfurt, Marie-Curie-Strasse 9, D-60439 Frankfurt, Germany, and the **Physiologisch-Chemisches Institut, Universität Tübingen, Hoppe-Seyler-Straße 1, D-72076 Tübingen, Germany

Addition of glucose to cells of the yeast Saccharomyces cerevisiae growing on a non-fermentable carbon source leads to selective and rapid degradation of fructose-1,6-bisphosphatase. This so called catabolite inactivation of the enzyme is brought about by the ubiquitin-proteasome system. To identify additional components of the catabolite inactivation machinery, we isolated three mutant strains, gid1, gid2, and gid3, defective in glucose-induced degradation of fructose-1,6-bisphosphatase. All mutant strains show in addition a defect in catabolite inactivation of three other gluconeogenic enzymes: cytosolic malate dehydrogenase, isocitrate lyase, and phosphoenolpyruvate carboxykinase. These findings indicate a common mechanism for the inactivation of all four enzymes. The mutants were also impaired in degradation of short-lived N-end rule substrates, which are degraded via the ubiquitin-proteasome system. Site-directed mutagenesis of the amino-terminal proline residue yielded fructose-1,6-bisphosphatase forms that were no longer degraded via the ubiquitin-proteasome pathway. All amino termini other than proline made fructose-1,6-bisphosphatase inaccessible to degradation. However, the exchange of the amino-terminal proline had no effect on the phosphorylation of the mutated enzyme. Our findings suggest an essential function of the amino-terminal proline residue for the degradation process of fructose-1,6-bisphosphatase. Phosphorylation of the enzyme was not necessary for degradation to occur.

In Saccharomyces cerevisiae cells growing on non-fermentable carbon sources, the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase)* is a long-lived protein with an approximate half-life of about 90 h (1). Shift of those cells to glucose-containing media leads to the selective and rapid degradation of this enzyme (half time of about 20 min (2)). This degradation process is called catabolite inactivation (3). A similar process was described for cytosolic malate dehydrogenase (cMDH) (4), isocitrate lyase (ICL) (5), and phosphoenolpyruvate carboxykinase (PEPCK) (6). In the case of FBPase, a rapid reversible phosphorylation is involved in the loss of enzymatic activity and followed by a proteolytic breakdown of the enzyme (1, 7, 8). Phosphorylation of FBPase was reported to shift the pH optimum of its activity from a neutral to a more alkaline pH (9). Also, the sensitivity of the enzyme to inhibition by the allosteric effectors AMP and fructose-2,6-bisphosphate is changed, but no effect on affinity of the enzyme to the substrate fructose-1,6-bisphosphate or the divalent metal activator Mg2+ could be measured (10). Phosphorylation of FBPase occurs at a serine residue in position 11 (11, 12). This modification was proposed to target the protein to the proteolytic machinery for degradation (7, 8). However, site-directed mutagenesis of the serine to an alanine residue, by this preventing phosphorylation, showed no effect and disproved its importance for inactivation (13).

Degradation of FBPase due to selective uptake into the vacuole and subsequent hydrolysis dependent on the vacuolar protease yscA has been reported (14). Other studies, however, identified the cytosolic proteasome as the main proteolytic system involved in catabolite inactivation of FBPase (15, 16). Ubiquitin conjugation was found to be an essential prerequisite for FBPase degradation (2), supporting degradation of the enzyme via the cytosolic proteasome. So far, the signals (degrons) determining FBPase for selective degradation after addition of glucose have not been identified in detail. However, glycolytic block mutants indicated that hexose phosphorylation was sufficient to trigger the proteolytic degradation (17). Using FBPase-β-galactosidase fusion proteins, the presence of instability determinants in at least two regions of FBPase could be shown (18).

Determination of galactosidase activities of ICL-β-galactosidase fusion proteins indicated the importance of a decapeptide sequence, located between amino acid residues 37 and 46 of ICL, for glucose-induced degradation of the enzyme (19). This decapeptide is not present in FBPase and other gluconeogenic enzymes.

Here, we describe the isolation of gid mutant cells, which are defective in glucose-induced degradation of FBPase. Character-

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This article is dedicated to the memory of Professor Dr. Dr. h. c. Helmut Holzer.

‡ These authors contributed equally to this work.

§ These authors are the senior authors of this work.

† Present address: Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.

¶ To whom correspondence should be addressed. Tel.: 49-711-685-4390; Fax: 49-711-685-4392; E-mail: dieter.wolf@po.uni-stuttgart.de.

1 The abbreviations used are: FBPase, fructose-1,6-bisphosphatase; cMDH, cytosolic malate dehydrogenase; PEPCK, phosphoenolpyruvate kinase; ICL, isocitrate lyase; ha, hemagglutinin; Ub, ubiquitin; PAGE, polyacrylamide gel electrophoresis; UFD, ubiquitin fusion degradation.

2 K. Köhn and K. D. Entian, unpublished data.
ization of these mutant strains suggests that they are also impaired in the breakdown of short lived N-end rule proteins (20), which are degraded via the ubiquitin-proteasome system (21). We therefore reasoned the involvement of a specific signal sequence at the amino terminus of FBPase necessary for degradation. A sequence alignment of gluconeogenic enzymes, which are subject to catabolite inactivation, like CdMDH and ICL, pointed to an amino-terminal proline residue that might be important for the degradation process. Site-directed mutagenesis of this proline residue yielded enzymatically active FBPase species, which are no more recognized by the catabolite inactivation machinery. Phosphorylation of FBPase is not a prerequisite for degradation.

EXPERIMENTAL PROCEDURES

Strains and Media—Yeast strains used in this work are listed in Table I. Cells were grown in mineral medium (MV), 0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% proline, 100 m ammonium sulfate, 2% ethanol, and required supplements. Radiolabeling for pulse-chase experiments was done in pulse medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% proline, 100 µM ammonium sulfate, 2% ethanol, and required supplements). Radiolabeling for phosphorylation experiments was done in phosphorylation medium (phosphate-free MV). In all experiments requiring induction of plasmid-encoded synthesis of ubiquitin, CuSO₄ was added to a final concentration of 100 µM.

Generation of Strains YMH1, YMH2, and YMH4—The linearized FBPase-S11A fragment was obtained by HindIII, SalI cleavage of plasmid pRV44 (13). The cleaved SalI site was obtained from the flanking vector sequence. The FBP1-P1W and FBP1-P1S was obtained by HindIII, SalI cleavage of pKD10W and pKD10S, respectively. The electro-phoretically purified fragments were used to transform the yeast strain Y303-1BKO. Transformants with an ethanol-resistant phenotype were selected, and the correct integration of the respective gene was confirmed by polymerase chain reaction using primers flanking the insertion sites.

Plasmids—The FBPase-β-galactosidase fusion containing plasmid pMZ1 derived from a DNA fragment coding for the amino-terminal part of the first 291 amino acids and ammonium sulfate, 0.5% proline, 100 µM ammonium sulfate, 2% ethanol, and required supplements. Radiolabeling for phosphorylation experiments was done in pulse medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% proline, 100 µM ammonium sulfate, 2% ethanol, and required supplements). Radiolabeling for phosphorylation experiments was done in phosphorylation medium (phosphate-free MV). In all experiments requiring induction of plasmid-encoded synthesis of ubiquitin, CuSO₄ was added to a final concentration of 100 µM.

Enzymatic Activities—Protein extracts from yeast cells were prepared as described by Ciriacy (29).

FBPase (30), ICL (31), MDH (32), PEPCK (33), and β-galactosidase (34) were measured as described. The protein concentration was determined using the microbiurct system (35). β-Galactosidase tests for mutant screen were as follows. Transformants containing the FBPase-β-galactosidase fusion protein were cultivated in 96-well microtiter plates. The wells were filled with 150 µl of synthetic complete medium with 2% glucose lacking uracil. Inoculation was done by transferring mutants with toothpicks. After 48 h of incubation at 28 °C under high humidity, the microtiter plates were centrifuged (2,000 × g, 5 min, room temperature). The medium was exchanged by a similar medium containing 2% glycerol and 2% ethanol instead of glucose. The cells were suspended again and incubated for another 4 days at 28 °C. The cultures were split to two equal aliquots by using a multichannel pipettor. One half was directly washed and frozen. After addition of glucose to a final concentration of 2%, the other half was incubated further for 100 min. The plates were centrifuged, washed twice with 0.1 M potassium phosphate buffer (pH 6.5), and frozen at −20 °C. Crude extracts were prepared in the microtiter plates by addition of 25 µl of Zymolyase 20T solution (Seikagaku, 0.5 mg/ml) to the thawed cell sediment in each well. After 30 min of incubation at 30 °C, the crude extract was used directly for determining β-galactosidase activity (36). The developed yellow dye was measured in a microtiter plate reader. Proteasome activities were measured in crude extracts, prepared in small scale according to Heinemeyer et al. (37). The three enzymatic activities of the proteasome were assayed as described by Fischer et al. (38).

Pulse-Chase Analysis and Immunoprecipitation—Pulse-chase experiments were done according to Schork et al. (2), using specific antibodies against FBPase. Protein bands were quantitated using a PhosphoImager storm (Molecular Dynamics).

Western Blot—Immunodetection of FBPase-ha-ubiquitin conjugates was done as described by Schork et al. (2).

Phosphorylation—Yeast strains were cultivated in MV medium containing 2% glucose and required supplements until an absorbance (A₅₆₄) of 0.6–0.7 was reached. After harvesting cells by centrifugation for 5 min at 5000 × g and washing, cells were preincubated for 4 h in MV medium containing 2% ethanol at an A₅₆₄ of 5. Cells were harvested by centrifugation (5 min at 5000 × g), washed with phosphorylation medium, and resuspended in phosphorylation medium at A₅₆₄ of 5. Radio-labeling was done by adding [32P]orthophosphate (Amersham, Braunschweig) to the cell suspension to a final concentration of 125 mCi per ml. After 30 min, a 1-ml sample was taken before glucose addition. Glucose was added to a final concentration of 2%, and a 1-ml sample was taken after 10 min of inactivation. Cell lysis and immunoprecipitation with specific FBPase antibodies was performed as described by Schork et al. (2).

RESULTS

Screen for gid Mutant Cells—To achieve a better understanding of the molecular mechanism of catabolite inactivation of FBPase, we generated mutants by ethylmethane sulfonate mutagenesis defective in the degradation of the enzyme.

A fusion protein consisting of the amino-terminal part (291AA) of FBPase linked to β-galactosidase was used to identify these mutants. Wild-type strain WAY 5-4A was trans-
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Inactivation Kinetics of Gluconeogenic Enzymes in gid Mutants—To characterize the mutant strains obtained, inactivation kinetics of FBPase and other gluconeogenic enzymes were followed (see “Experimental Procedures”). Within 150 min after glucose addition, the three gid mutants showed only a minor decrease of FBPase activity, which was the result of FBPase phosphorylation (Fig. 1A).

Catabolite inactivation in S. cerevisiae has also been described for PEPCK (39), cMDH (40), and ICL (41). To test if the gid mutants were generally affected in catabolite inactivation, we followed the inactivation kinetics of these enzymes in addition to FBPase. All three mutants also revealed, besides a defect in the inactivation of FBPase, an impaired inactivation of PEPCK, cMDH, and ICL (Fig. 1A). Three MDH isoenzymes are expressed in S. cerevisiae, but only the cMDH is a target of catabolite inactivation. Therefore, MDH activity in wild-type cells even 150 min after glucose addition does not decrease beyond 40% of the initial activity (Fig. 1A) (40).

Pulse-Chase Analysis of gid Mutant Cells—To confirm that the defective inactivation of FBPase found in gid mutants is due to defective degradation, we examined the mutants in a pulse-chase experiment. Wild-type and gid mutant strains were radiolabeled with [35S]methionine, derepressed for FBPase on ethanol-containing medium, and transferred onto glucose medium to induce FBPase degradation. Samples were taken at different time points, cells were lysed, and radiolabeled FBPase was immunoprecipitated using specific antibodies, separated on SDS-PAGE and visualized by autoradiography. After 1 h on glucose, FBPase protein is almost completely degraded in wild-type cells, whereas in gid mutant strains FBPase is visible even after 2 h (Fig. 1B).

The 20 S Proteasome Activities Are Unaffected in gid Mutant Cells—Mutants defective in proteolytic activities of the yeast proteasome had revealed the involvement of this protease complex in glucose-induced degradation of FBPase (15, 16), indicating that this process is a cytoplasmic event. To check if defective degradation in the gid mutant cells is due to impaired proteolytic activity of the 20 S proteasome, crude extracts of stationary phase-grown gid cells were assayed using three different peptide substrates to trace the three active sites of the enzyme complex (42). No significant decrease in the respective activities of the 20 S proteasome compared with the isogenic wild-type were found (Fig. 2).

Stabilization of N-end Rule Substrates in gid Mutant Cells—Degradation of FBPase occurs via the ubiquitin pathway (2). To elucidate a possible involvement of the mutated gid proteins in the ubiquitin pathway, we measured the steady-state levels of short-lived N-end rule substrates, known to be degraded via the ubiquitin proteasome pathway, in the gid strains. We used
three different ubiquitin-β-galactosidase fusion proteins: Pro-β-gal, Leu-β-gal, and Arg-β-gal (20). In contrast to all other ubiquitin-β-galactosidase fusion proteins, Pro-β-gal is slowly deubiquitinated. Although the fusion protein is cleaved with a short half-life, deubiquitinated Pro-β-gal is rather stable (20).

The steady-state level of the Ub-Pro-β-gal fusion is increased 2-fold only in gid3–1 cells (Table II). Galactosidase activity of cells expressing Leu-β-gal in gid mutant cells is similar to the value found in proteasome mutant cells, about 10-fold higher as compared with isogenic wild-type cells. The highest increase of galactosidase activity was observed in cells expressing the very short-lived Arg-β-gal fusion. Steady-state levels of Arg-β-gal was increased 27-fold in gid1–1, 48-fold in gid2–1, and 21-fold in gid3–1 mutant cells, respectively. For comparison, a 13.6-fold increase of this activity was observed in proteasomal pre1–1 pre2–1 double mutant cells (21).

Site-directed Mutagenesis of the Amino-terminal Proline Prevents FBPase Degradation—The gluconeogenic enzymes FBPase, cytosolic malate dehydrogenase, and ICL are all subject to rapid glucose-induced degradation. A sequence alignment pointed to an amino-terminal proline residue as a common feature of these enzymes (Fig. 3A). Interestingly, this proline residue is lacking in FBPase of Saccharomyces pombe and E. coli, two FBPases that are not targets of glucose-induced breakdown. Using site-directed mutagenesis, we exchanged the amino-terminal proline of FBPase by all other 19 amino acids. This exchange resulted in a decrease of FBPase activity of 3–5 fold in FBPase or Trp1-FBPase (Fig. 3B). Site-directed mutagenesis of the amino-terminal proline prevented enzyme degradation.

Glucose-induced Polyubiquitination of FBPase Depends on Its Amino-terminal Proline Residue—Polyubiquitination of FBPase upon glucose addition is a prerequisite for degradation of the enzyme to occur (22). Therefore, we tested whether the amino-terminally mutated FBPase species Ser1-FBPase and Trp1-FBPase were still targets of the ubiquitination machinery.

Cells expressing the different forms of FBPase, transformed with plasmid YEp112 expressing haUb, were derepressed on ethanol. After addition of glucose, samples were taken at different time points. Crude extracts were immunoprecipitated with FBPase antibodies, and proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters. Filters were then probed with ha antibodies. Neither in cells expressing Ser1-FBPase nor in cells expressing Trp1-FBPase were any ubiquitin conjugates detectable (Fig. 4). In contrast, polyubiquitination was visible for FBPase, which carried the mutated phosphorylation site serine into alanine as well for the wild-type control.

Phosphorylation of FBPase during Catabolite Inactivation—The first step after addition of glucose to yeast cells grown on a non-fermentable carbon source rests in phosphorylation of the enzyme. We therefore examined the phosphorylation event in the different mutant FBPase versions. Cells expressing wild-type FBPase or mutant FBPases, respectively, were labeled with [32P]orthophosphate during derepression of the enzyme. After inducing the inactivation reaction by addition of glucose,
samples were taken. Crude extracts were immunoprecipitated with FBPAse antibodies, proteins were separated by SDS-PAGE, and phosphorylated protein was analyzed by autoradiography. As can be seen in Fig. 5, both mutant FBPAses were phosphorylated like the wild-type enzyme.

As expected, only the Ala11-FBPAse was not phosphorylated after the addition of glucose.

The Ubiquitin Fusion Degradation Pathway Is Not Involved in the Degradation of FBPAse—Proteolysis of the N-end rule substrate ubiquitin-Pro-β-galactosidase via the ubiquitin-mediated pathway requires formation of a multitiubiquitin chain by the ubiquitin fusion degradation pathway (UFD) (43). This pathway links the multitiubiquitin chain to the amino-terminal proline of the substrates. Our finding that catabolite inactivation of FBPAse is dependent on the amino-terminal proline directed us to the question if the UFD is also connected to the degradation of substrates carrying a free amino-terminal proline. We therefore measured the degradation rate of FBPAse after addition of glucose in yeast strains defective in the sec63 genes. The function of the other SON1 ufld3 genes after addition of glucose in yeast strains defective in the sec63 alleles. We therefore measured the degradation rate of FBPAse after addition of glucose in yeast strains defective in the sec63 genes ufld1, ufld2, ufld3, and ufld5. The UFDS gene is identical to SON1, an extragenic suppressor of a ts growth defect of certain sec63 alleles. The function of the other UFDS genes is so far unknown. Immunoblot analysis indicated an unaffected glucose-induced degradation in all the ufld mutants (not shown).

The same result was obtained with a ufld4 deletion strain (Fig. 6) defective in a member of the E6AP family of ubiquitin ligases.

**DISCUSSION**

Signals leading to specific, rapid degradation of proteins are only very poorly understood. The catabolite inactivation pathway signaling selective degradation of FBPAse represents an ideal model to study such a process. To get insight into this process, we isolated gid mutants defective in glucose-induced degradation of FBPAse. They fell into three complementation groups. The gid1, gid2, and gid3 mutant cells exhibited a defect not only in degradation of FBPAse but also in inactivation of cMDH, ICL, and PEPCK (Fig. 1), other enzymes subject to catabolite inactivation.

As FBPAse is inactivated via the proteasome, we checked for a defect in this enzyme complex using chromogenic peptide substrates, testing the three prominent proteasomal activities. These activities seemed to be unaffected, suggesting a wild-type-like proteolytic capacity of the proteasome in gid mutant strains (Fig. 2).

Analysis of several N-end rule substrates, ubiquitin-X-β-galactosidase fusion proteins in gid mutant cells, unraveled a drastic stabilization especially of the short-lived Arg-β-galactosidase especially of the short-lived Arg-β-galactosidase fusion proteins in sec63 alleles. The function of the other SON1 ufld3 genes after addition of glucose in yeast strains defective in the sec63 alleles.
these mutated FBPase species after glucose addition (Fig. 3). The N-end rule would predict Trp1-FBPase to be a short-lived protein and Ser1-FBPase to be a more long-lived one (20).

Interestingly, glucose addition to cells abolished catabolite inactivation of both FBPase mutant forms. They exhibited a similar stability (Fig. 3, B and C). This supports the essential role of Pro1 for the recognition of FBPase by the catabolite inactivation machinery.

In a previous study, polyubiquitin conjugation was found as to be a prerequisite for FBPase degradation after addition of glucose (2). Replacing Pro1 of FBPase by other amino acids also prevented the polyubiquitination of these modified forms (Fig. 4), indicating the lack of the signal for the ubiquitinating machinery.

Phosphorylation of FBPase was not affected in our amino-terminal point-mutated species (Fig. 5), supporting the idea that this covalent modification is essential for rapid inactivation of the enzymatic activity but not for degradation. This is further supported by the fact that exchange of the serine residue 11 of FBPase (which is the target of phosphorylation) to alanine does not prevent catabolite degradation of the enzyme.

After addition of glucose to cells, we expect the recognition of the amino-terminal proline of FBPase by specific protein components, which lead to polyubiquitination of the protein and subsequent degradation via the cytosolic 26 S proteasome.

The ubiquitin-conjugating enzymes Ubc4 and Ubc5 are necessary for the ubiquitination of FBPase and the N-end rule substrate ubiquitin-Pro-β-galactosidase (2, 43). We examined the involvement of ufd mutants, which are responsible for the degradation of ubiquitin-pro-β-galactosidase fusion protein, in catabolite degradation of FBPase but found no effect. The specificity of the ubiquitin system for a certain protein is thought to be a property of a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). However, the ubiquitin ligase, Ufd4, was not involved in this process. Obviously, the cell differentiates between a ubiquitin-linked amino-terminal proline and a free amino-terminal proline in proteins. This suggests the existence of additional ubiquitin ligases specific for the selective proteolysis of gluconeogenic enzymes. Further work will be needed to identify additional components of the catabolite inactivation system.

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