Catabolite Inactivation of Fructose-1,6-bisphosphatase of Saccharomyces cerevisiae

DEGRADATION OCCURS VIA THE UBIQUITIN PATHWAY*

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Catabolite inactivation of fructose-1,6-bisphosphatase (FBPase), a key enzyme in gluconeogenesis, is due to phosphorylation and subsequent degradation in the yeast Saccharomyces cerevisiae. The degradation process of the enzyme had been shown to depend on the action of the proteasome. Here we report that components of the ubiquitin pathway target FBPase to proteolysis. Upon glucose addition to yeast cells cultured on nonfermentable carbon sources FBPase is ubiquitinated in vivo. A multibiquitin chain containing isopeptide linkages at Lys48 of ubiquitin is attached to FBPase. Formation of a multibiquitin chain is a prerequisite for the degradation of FBPase. Catabolite degradation of FBPase is dependent on the ubiquitin-conjugating enzymes Ubc1, Ubc4, and Ubc5. The 26 S proteasome is involved in the degradation process.

Specific and rapid degradation of certain proteins is a fundamental mechanism in many biological processes, including embryonic development, cell proliferation, cell cycle control, and metabolic regulation (1). The pathways signaling selective proteolysis are only very poorly understood. The regulation of FBPase, a key enzyme in gluconeogenesis, represents an ideal model to study such signaling pathways. The cytoplasmic enzyme is subject to catabolite inactivation in the yeast Saccharomyces cerevisiae (2). Addition of glucose to yeast cells grown on nonfermentable carbon source causes a rapid inactivation of FBPase due to phosphorylation and subsequent degradation (3–5). Since its discovery (6), the proteolytic mechanism of FBPase degradation had remained elusive. The prominent proteolytic activities of the vacuole, especially proteinase yscB, in degrading FBPase in vitro at first suggested this lysosomal enzyme to be the catalyst of FBPase degradation under catabolite inactivation conditions (7). However, experiments using mutants defective in the activity of proteinase yscB and other vacuolar proteases seemed to rule out this possibility (8–10). Recently, selective uptake of FBPase into the vacuole and degradation by vacuolar proteases under catabolite inactivation conditions had been proposed (11). However, mutants defective in proteolytic activities of the yeast proteasome revealed the involvement of this protease complex in glucose-induced degradation of FBPase, indicating this process to be a cytoplasmic event (12, 13). 26 S proteasomes are large multicatalytic protease complexes located in the cytoplasm and nucleus of the eukaryotic cell. The 26 S proteasome is build up from a 20 S proteasome core and two additional 19 S subcomplexes (14, 15). Proteasome mutants demonstrated the involvement of the enzyme complex in stress-dependent and ubiquitin-mediated proteolysis (16–18). In vitro studies demonstrated that 26 S proteasomes rather than 20 S proteasomes are able to degrade ubiquitinated proteins in an ATP-dependent fashion (19–21).

In eukaryotes the ubiquitin system constitutes the major cytoplasmic pathway targeting proteins to selective degradation (22–26). Ubiquitin, a 76-residue polypeptide found in all eukaryotes, is covalently attached with its carboxyl-terminal glycine to the protein substrate through an isopeptide bond in a multistep reaction. Ubiquitin is first activated through the formation of a thiolester bond of the carboxyl-terminal glycine with the ubiquitin-activating enzyme E1. Thereafter ubiquitin is transferred to a ubiquitin-conjugating enzyme E2, which subsequently links it to the ε-amino group of an internal lysine of the substrate protein. In some cases this last step is performed with the support of a ubiquitin ligase E3, which mediates the recognition of the target protein together with the E2 enzyme. In further successive reactions ubiquitin moieties are linked to the Lys48 residue of each previously conjugated ubiquitin molecule until a polyubiquitin chain is formed (27). The specificity of the ubiquitin system for a certain substrate is thought to be a property of the E2 and E3 enzymes.

In yeast, 10 UBC (E2) genes have been identified until now (23). UBC1, UBC4, and UBC5 encode a functionally overlapping group of enzymes, which mediate bulk turnover of short-lived and abnormal proteins (28, 29). The UBC6 gene product resides in the endoplasmic reticulum membrane and seems to be involved in degradation of endoplasmic reticulum membrane proteins (30). UBC3 (CDC34) and UBC9 encode genes required for degradation of cyclins and regulation of the cell cycle (31, 32). The UBC2 (RAD6) gene is involved in different processes including DNA repair and protein degradation via the N-end rule pathway (33, 34). The UBC10 (PAS2) gene product is essential for the biogenesis of peroxisomes (35). UBR1 is the only gene encoding an E3 enzyme that has been identified in yeast as yet (36).

Only a few in vivo substrates have been known up to now, which are targeted to degradation by the ubiquitin system. They include the yeast transcriptional regulators Matα2 (37) and GCN4 (38), cyclins (33, 39–41), and the oncogene products p53 (42) and Msv41 (43).

Another system rendering proteins susceptible to specific degradation via the proteasome is antizyme. Its function in the degradation of the tightly controlled ornithine decarboxylase has been shown in vitro and in vivo (44, 45).
The signal rendering FBPase accessible to proteolysis through the proteasome during the catabolite inactivation process had remained unknown. Here we show that glucose-induced degradation of FBPase is signalised via the ubiquitin pathway.

MATERIALS AND METHODS

Plasmids—YPEp96 is a 2μm S. cerevisiae Escherichia coli-based shuttle vector that encodes a synthetic version of yeast ubiquitin (Ub) under the control of the copper-inducible CUP1 promoter. YEp110 is identical to YEp96, with the difference that instead of wild-type Ub it encodes yeast Ub with an arginine (instead of lysine) at amino acid position 48 (Ub-R48). YEp112 codes for Ub with an epitope (hemagglutinin (ha) of influenza virus attached to the amino terminus (haUb). Except for this modification, the plasmid is also identical to YEp96. The plasmids used for expression of the different ubiquitin variants were a gift from M. Hochstrasser (46).

Strains and Media—For overexpression of the different Ub variants, the S. cerevisiae wild-type strain W3031B (MATα ade2-1 arg1-2-3 his3-11,15 trp1-1 ura3-1) and the FBPase deletion mutant strain W3031B-K0 (MATα ade2-1 leu2-3,11 his3-11 trp1-1 ura3-1 fbp1::LEU2) were transformed with plasmid YEp96, YEp110, or YEp112, respectively. Both strains were a gift from H.-L. Chiang (11). UbC mutant strains used in this study are congenic to wild-type strain YW01: ubc4::HIS3, ubc5::LEU2, ubc6::HIS3, ubc7::LEU2, ubc8::HIS3, ubc9::LEU2. YEp112 was a gift from H.-L. Chiang (11). Strain CMY762 (MATa dim3-1 ura3-52 leu2Δ1 his3Δ3-200) and congenic wild-type strain YPH499 were gifts from D. Herskowitz (55).

RESULTS AND DISCUSSION

Ubiquitin-mediated degradation of a protein requires the covalent attachment of ubiquitin moieties, leading to the occurrence of higher molecular mass species of such a protein. The use of an epitope-tagged ubiquitin derivative allows the unambiguous identification of such ubiquitin protein conjugates in vivo (46, 48).

If catabolite degradation of FBPase were mediated via the ubiquitin pathway, higher molecular mass species of FBPase would be expected. When pulse-labeled FBPase was immunoprecipitated during catabolite inactivation in cells expressing wild-type ubiquitin from a 2μm plasmid, at least two additional labeled species with higher mass than FBPase could be detected in the precipitates (Fig. 1A, lanes 2 and 3). The appearance of these larger species was only visible after shifting cells derepressed FBPase onto glucose medium (compare Fig. 1A, lane 1 with lanes 2 and 3), suggesting that these species are intermediates of the glucose-induced degradation process of FBPase. To test whether these species actually represent ubiquitin conjugates, a ha-tagged version of ubiquitin was overexpressed. This ubiquitin variant (haUb) is about 1.5 kDa (14 amino acids) larger than wild-type ubiquitin, resulting in a decrease of the mobility of this molecule on SDS-PAGE.

If the larger protein species present in the FBPase precipitate (Fig. 1A) were ubiquitin-conjugated, then reversion of wild-type ubiquitin with haUb would lead to a slower migration of these species as compared with FBPase ligated to wild-type ubiquitin.

Cells transformed with either plasmid YEp96 expressing wild-type Ub or plasmid YEp112 expressing haUb were pulse-labeled during derepression of FBPase. After inducing the activation period by addition of glucose, samples were taken at 15-min intervals with PBS-T buffer (50 mM K2HPO4, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 10% nonfat milk. The filter was blocked by gentle shaking for 3 h at room temperature with PBS-T buffer (50 mM K2HPO4, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 10% nonfat milk. The filter was then treated with ha antibodies (1:5000 in PBS-T) and incubated three times with a 1:5000 dilution of anti-mouse IgG, respectively.

The use of an epitope-tagged ubiquitin derivative allows the unambiguous identification of such ubiquitin protein conjugates in vivo (46, 48).

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Catabolite Inactivation of FBPase of S. cerevisiae

Fig. 1. FBPase is ubiquitinated in vivo. A, pulse-chase analysis of FBPase degradation. In addition to FBPase, FBPase immunoprecipitates show a set of higher molecular mass species (arrows, lanes 2 and 3). Cells of W3031B overexpressing wild-type Ub were pulse-labeled during derepression of FBPase in ethanol-containing medium and chased with the addition of glucose, followed by extraction, immunoprecipitation, and SDS-PAGE as described under “Materials and Methods.” B, comparison of the SDS-PAGE pattern of FBPase immunoprecipitates from labeled W3031B cells expressing either wild-type ubiquitin (wt-Ub, lane 2) or haUb (lane 3). Expression of haUb in W3031B cells leads to an increase of the apparent molecular masses of the putative Ub-FBPase conjugates. FBPase null mutant (Δfbp1) cells expressing either wild-type Ub or haUb show no corresponding ubiquitin conjugates (lanes 1 and 4). Samples were taken 20 min after the addition of glucose to the cells.

Fig. 2. Immunodetection of Ub-FBPase conjugates during catabolite inactivation. Crude extracts from cells expressing either haUb or wild-type ubiquitin (wt-Ub) were immunoprecipitated with FBPase antibodies and separated by SDS-PAGE. Thereafter the proteins were transferred to a nitrocellulose membrane and probed with ha antibodies. A variety of different haUb-FBPase conjugates occur after shifting the cells onto glucose (lanes 6–8). The amount of these conjugates decline with increasing time after glucose addition (lanes 6–8). Expression of haUb in W3031B cells leads to an increase of the apparent molecular masses of the putative Ub-FBPase conjugates. FBPase null mutant (Δfbp1) cells expressing either wild-type Ub or haUb show no corresponding ubiquitin conjugates (lanes 1 and 4). Samples were taken 20 min after the addition of glucose to the cells.

Complex spectrum of ha-ubiquitin-FBPase conjugates after shifting cells to glucose. The amount of ubiquitinated FBPase species is highest shortly after glucose addition (Fig. 2, lane 6), followed by a drastic decline along with further incubation of the cells on glucose (Fig. 2, lanes 7 and 8). This behavior is consistent with the view that the ubiquitinated forms of FBPase are the substrates of the degradation machinery of the cell. As expected, neither in cells expressing wild-type Ub nor in FBPase null mutant cells (Δfbp1) expressing haUb is any ubiquitin conjugate detectable (Fig. 2, lanes 2 and 4 respectively), clearly confirming that the observed bands in Fig. 2 (lanes 6–8) represent ubiquitinated forms of FBPase. From these data we conclude that FBPase is ubiquitinated upon glucose addition and that this event triggers catabolite degradation of the enzyme.

It has been shown that treatment of cells with cycloheximide at the time of glucose addition prevents degradation of FBPase (11). We found that FBPase is also not ubiquitinated under these conditions (not shown). This indicates the necessity upon catabolite inactivation of the new synthesis of a protein(s) that is part of the signaling cascade and indispensable for ubiquitination and degradation of FBPase.

For the N-end rule substrates (27) and for the Matα2 repressor (46) it has been shown that proteolysis via the ubiquitin-mediated pathway requires formation of a mult ubiquitin chain for their efficient degradation. The ubiquitin molecules are linked within this chain by isopeptide bonds connecting the carboxyl-terminal Gly of one ubiquitin moiety to the ε-amino group of Lys of the adjacent ubiquitin molecule. Lys(s) and Lys(s) have also been found to be sites for polyubiquitination (49), but Lys has been identified as the primary site of this process. A modified ubiquitin carrying an arginine at position 48 (Ub-R48) instead of lysine can still be conjugated to other proteins but fails to function as an acceptor within the mult ubiquitin chain (27). Therefore, Ub-R48 can serve as a probe for monitoring the presence of a Lys-linked mult ubiquitin chain in a protein of interest (6).

Western blot analysis had indicated the appearance of multiply ubiquitinated FBPase molecules during catabolite inactivation (Fig. 2, lane 6). If Lys(s)-linked mult ubiquitination is a prerequisite for glucose-induced degradation of FBPase, the presence of Ub-R48 should affect this process. To address this possibility we compared the degradation rate of FBPase in cells expressing high levels of wild-type ubiquitin (transformed with YEp96) and in cells expressing high levels of Ub-R48 (transformed with YEp110) by pulse-chase analysis. Although glucose-induced degradation of FBPase occurred in cells overexpressing wild-type ubiquitin (Fig. 3A), the presence of Ub-R48 inhibited the degradation of the enzyme (Fig. 3B). It is noticeable that the expression of Ub-R48 resulted in an increase of the amount of the monoubiquitinated FBPase species (see arrow in Fig. 3). The SDS-PAGE pattern of FBPase immunoprecipitates from pulse-labeled cells expressing Ub-R48 did not show multiply ubiquitinated species of FBPase (not shown). Formation of a mult ubiquitin chain containing Lys(s) linkages seems therefore to be necessary for glucose-induced degradation of FBPase. As degradation of FBPase is nearly
blocked in cells expressing Ub-R48, Lys48 must be viewed as the important site for polyubiquitination to render FBPa accessible to proteolysis.

Using mutants defective in subunits of the 20 S proteasome we had shown that this particle is involved in catabolite degradation of FBPa (12–13). In vitro studies had shown that ubiquitinated proteins are degraded by the 26 S proteasome (19–21). To elucidate whether the 26 S complex is needed for FBPa degradation, we assayed the catabolite inactivation of the enzyme in strains carrying a regulatory subunit of the 26 S proteasome (47). Cim3-1 mutant cells arrest the cell cycle at nonpermissive temperature and accumulate Ub-Projigal, degradation of which is ubiquitin-dependent. Pulse-chase analysis of FBPa during glucose-induced degradation indicated that the enzyme is remarkably stabilized in cim3-1 cells relative to the isogenic wild type (Fig. 4). This documents that the entire 26 S proteasome complex is necessary for catabolite inactivation to occur.

The finding that catabolite inactivation of FBPa is triggered by ubiquitination pointed to the action of E2 (Ubc) enzymes involved in this process. Therefore we measured the degradation rate of FBPa after the addition of glucose in yeast strains deleted in various Ubc genes and thus devoid of the respective activity of the Ubc enzyme. Pulse-chase analysis indicated that catabolite degradation of FBPa was unaffected in cells lacking a functional Ubc2 (Rad6), Ubc6, Ubc7, and Ubc10 (Pas2) protein (not shown). In contrast, the degradation was strongly inhibited in ubc1 and ubc4 ubc5 mutant strains (Fig. 5A). The half-life of FBPa was increased 2-fold in ubc1 cells and 4-fold in ubc4 ubc5 double mutant cells (Fig. 5B). Single deletions of either UBC4 or UBC5 led only to a weak but reproducible increase of the half-life of FBPa during the catabolite inactivation process (25 min and 20 min, respectively, versus 17 min in wild-type cells, not shown). This behavior might have been expected, because it is conceivable that the nearly identical Ubc4 and Ubc5 enzymes are functionally redundant (28–29).

When measuring the degradation of FBPa by assaying enzymatic activity, similar results were obtained (not shown). It should be noted that all ubc mutants showed the characteristic rapid loss of FBPa activity of about 50% during the first 15 min of catabolite inactivation. This is due to phosphorylation of FBPa (4–5) and indicates that the transduction of the glucose-induced signal is not impaired in the ubc mutant cells. On the basis of genetic studies, it had been shown that Ubc1, Ubc4, and Ubc5 constitute a subfamily of ubiquitin-conjugating enzymes with overlapping functions (28). This feature is nicely reflected in the catabolite degradation process of FBPa.

Based on the above described findings we assume the following model for catabolite inactivation of FBPa. The addition of glucose to yeast cells grown on a nonfermentable carbon source causes phosphorylation (4–5) and ubiquitination of the enzyme.
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