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The ubiquitin ligase SCF^{Grr1} is required for Gal2p degradation in the yeast *Saccharomyces cerevisiae*

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Abstract

F-box proteins represent the substrate-specificity determinants of the SCF ubiquitin ligase complex. We previously reported that the F-box protein Grr1p is one of the proteins involved in the transmission of glucose-generated signal for proteolysis of the galactose transporter Gal2p and fructose-1,6-bisphosphatase. In this study, we show that the other components of SCF^{Grr1}, including Skp1, Rbx1p, and the ubiquitin-conjugating enzyme Cdc34, are also necessary for glucose-induced Gal2p degradation. This suggests that transmission of the glucose signal involves an SCF^{Grr1}-mediated ubiquitination step. However, almost superimposable ubiquitination patterns of Gal2p observed in wild-type and *grr1Δ* mutant cells imply that Gal2p is not the primary target of SCF^{Grr1} ubiquitin ligase. In addition, we demonstrate here that glucose-induced Gal2p proteolysis is a cell-cycle-independent event.

Keywords: SCF^{Grr1} ubiquitin ligase; Gal2 transporter; Catabolite degradation; Yeast

Like other eukaryotic microorganisms, the budding yeast *Saccharomyces cerevisiae* must be able to respond rapidly to varying environments in its natural habitat in order to grow and proliferate appropriately. To achieve optimal exploitation of available nutrients, the yeast developed a complex regulatory network which commonly operates both at the level of gene transcription and/or at the posttranslational level. A well-known example of posttranslational regulation of activities of some key gluconeogenic enzymes and some sugar-specific transporters in response to an easily fermentable carbon source is a mechanism known as catabolite degradation or catabolite inactivation [1,2]. The heart of the mechanism lies in a glucose-induced ubiquitination of the target proteins which, in turn, triggers their degradation through the 26S proteasome (in the case of key enzymes of gluconeogenesis) or by the action of vacuolar proteolysis (in the case of sugar-specific transport proteins). We have previously reported that glucose addition to the galactose-grown cells triggers monoubiquitination of the galactose transporter Gal2p at several lysine residues, followed by its release from the plasma membrane and transfer via the endocytotic system to the vacuole for proteolysis [3,4]. In contrast, upon glucose addition to cells grown on media containing a non-fermentable carbon source like ethanol or glycerol, polyubiquitination of fructose-1,6-bisphosphatase (FBPase), the key enzyme of gluconeogenesis, leads to its degradation by the 26S proteasome [5–8]. Furthermore, examination of the mechanism that determines the different fates of Gal2p and FBPase observed during their catabolite degradation uncovered that the systems responsible for their proteolysis, share protein components of the same glucose signaling pathway [9]. Indeed, initiation of both, Gal2p and FBPase proteolysis appears to require the rapid transport of glucose and/or those glucose-related sugars that are at least partly

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metabolized, e.g., phosphorylated, by hexokinase Hxk2p. The yet unknown metabolite signal generated in this way is subsequently transmitted to the targets through downstream components of the glucose signaling pathway, including Reg1 and Grr1p [9].

In this study, we demonstrate that Grr1p plays a role in Gal2p catabolite degradation in the form of the SCF^{Grr1} complex. We show strong stabilization of Gal2p in *cdc34*, *skp1*, and *rbx1* (but not in *cdc53*) mutant cells under the conditions of catabolite degradation, indicating that the Cdc34, Skp1, and Rbx1 proteins are required for this process as is Grr1p. However, similarity-if not identity—of ubiquitination patterns of Gal2p observed in wild-type and *grr1Δ* mutant cells suggests that the role of SCF^{Grr1} in Gal2p degradation is most likely indirect. We also show that Gal2p degradation is an event which proceeds independently of cell-cycle progression.

Materials and methods

Strains, growth conditions, and methods. The genotype and source of the *S. cerevisiae* strains used in this study are given in Table 1. Strains are derived from W303: *MATa*,*ade2-1*, *ura3*, *leu2-3*,*112*, *his3-1*,*15*, *trp1-1*, and *can1-100*, or BY4743: *Mata*/α,*his3Δ1*/*his3Δ1*, *leu2Δ0*/*leu2Δ0*, *MET15*/*met15Δ0*, *lys2Δ0*/*LYS2*, and *ura3Δ0*/*ura3Δ0*. In strains used for detection of Gal2p ubiquitination, wild-type ubiquitin (Ub) and Myc-tagged ubiquitin (Myc-Ub) were expressed from the P_{CUP1} promoter in high-copy plasmids YEp96 and YEp105, respectively. Both plasmids were a kind gift from Hochstrasser [10]. Yeast cells were cultured in rich media (1% yeast extract, 2% bacto peptone, and 50 μM/L adenine) or synthetic media (0.67% yeast nitrogen base without amino acids supplemented with auxotrophic requirements) in the presence of the appropriate 2% carbon source. Except for temperature-sensitive strains, all yeast strains were grown at 30 °C to an early exponential phase and further treated at the same temperature. Strains carrying temperature-sensitive alleles were grown at 25 °C and thereafter incubated at 37 °C for different time intervals, depending on the strain used. To induce Gal2p inactivation, the cells were washed with water and resuspended in 0.17% yeast nitrogen base without ammonium sulfate and amino acids plus 2% glucose. After inactivation, the cells were collected by centrifugation, washed with water, and assays of Gal2p degradation were performed as previously described [4,9]. Crude cell extracts were prepared either by a TCA procedure or by glass bead lysis. The proteins were resolved by SDS–PAGE, and Western blot analyses of Gal2p were carried out as described previously [4,9]. Gal2p blotted onto nitrocellulose sheets was detected using polyclonal anti-Gal2p antibody (1:2000) and peroxidase-coupled goat anti-rabbit antibody (1:10,000, Medac, Germany). Antibodies were detected using enhanced chemiluminescence (ECL) with ECL or ECL Plus reagents (Amersham Biosciences, Germany). The relative intensities of Gal2p bands at each time point were quantified by scanning densitometry of ECL-films.

Synchronization of cells. Exponentially grown wild-type cells were synchronized in early G_1 phase with the mating pheromone α-factor (3 μM final concentration), in early S-phase with hydroxyurea (130 mM final concentration), and in metaphase with nocodazole (50 μM). Incubation at OD_{600} of 0.4–0.5 and 30 °C was performed until more than 90% of cells were unbudded (for α-factor) or budded (for hydroxyurea and nocodazole arrest). Synchronization of *cdc28-4* and *cdc15-2* mutant strains in G_1 phase and telophase, respectively, was achieved by shifting 25 °C grown cells to 37 °C for 4 h at an OD_{600} of 0.4–0.5.

Detection of ubiquitinated Gal2p. This method was adopted from Jarosch et al. [11]. Wild-type and *grr1Δ* mutant cells transformed with a plasmid expressing Ub and/or Myc-Ub were grown on synthetic medium containing 2% raffinose until an OD_{600} of 0.5–1.0 was reached. After harvesting and washing, the cells were incubated in the same medium in the presence of 2% galactose and 100 μM $CuSO_4$ at 25 °C for 4 h and then shifted to 37 °C for additional 2 h. The cells were harvested by centrifugation, and samples (20 OD_{600}/ml) were taken before and 30 min after addition of inactivation medium. The cell sediments were washed once with ice-cold 20 mM sodium azide containing 2 mM PMSF and 20 mM *N*-ethylmaleimide (NEM) and once with sorbitol buffer (SB buffer; 0.7 M sorbitol, 50 mM Tris–HCl, pH 7.5) with 2 mM PMSF and 20 mM NEM. Subsequently, all material was kept on ice. Cells were mechanically lysed using glass beads in ice-cold SB buffer containing 2 mM PMF, and 20 mM NEM and protease inhibitors (Complete, Roche Diagnostics, Germany) for 20 min at 4 °C on a multivortexer. Lysates were cleared by centrifugation at 3500 rpm for 5 min and the crude membrane fraction was then prepared by centrifugation of the supernatant at 14,000 rpm for 1 h and 4 °C. The pellet was resuspended in 100 μl of solubilization mixture (50 mM Tris–HCl, pH 6.8, containing 8 M urea, 5% SDS, and 0.1 mM EDTA) and 3% dithiothreitol. The samples were incubated at 37 °C for 30 min and diluted in 1 ml of cold immunoprecipitation buffer (IP buffer; 50 mM Tris–HCl, pH 7.5, 190 mM NaCl, 6 mM

Table 1
Yeast strains used in this study

Strain name	Relevant genotype	Background	Source
K699	Wild-type	W303	M. Tyers
YMT670	*cdc34-2*	W303	M. Tyers
YMT871	*cdc53-1*	W303	M. Tyers
Y80	Wild-type	W303	S. Elledge
Y552	*skp1-11*	W303	S. Elledge
Y554	*skp1-12*	W303	S. Elledge
rbx1-1	*rbx1*::*HIS3* × PDK102 (*rbx1-1* in pRS314)	W303	S. Elledge
YHY284	*grr1*::*LEU2*	W303	C. Wittenberg
H192	*gal2*::*HIS3*	W303	H. Ronne
Y34633(*cul3Δ*)	*ygr003w*::*KANMX4*/*ygr003w*::*KANMX4*	BY4743	Euroscarf
Y31376(*cul8Δ*)	*yjl047c*::*KANMX4*/*yjl047c*::*KANMX4*	BY4743	Euroscarf
Y38902(*grr1Δ*)	*yjr090c*::*KANMX4*/*yjr090c*::*KANMX4*	BY4743	Euroscarf
Y32692(*gal2Δ*)	*ylr081w*::*KANMX4*/*ylr081w*::*KANMX4*	BY4743	Euroscarf