Ubiquitin and the SCF^{Grr1} ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in *Saccharomyces cerevisiae*

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Abstract In response to external amino acids, the permease-like sensor Ssy1 of *Saccharomyces cerevisiae* activates a pathway leading to transcriptional induction of several permease genes including *AGP1* and *PTR2*. We previously reported that *AGP1* induction requires Grr1, the F-box protein part of the SCF^{Grr1} ubiquitin–ligase complex. We show here that ubiquitin, other components of SCF^{Grr1} and the ubiquitin-conjugating enzyme Cdc34 are essential for *AGP1* and *PTR2* induction. This suggests that transduction to these genes of the amino acid signal generated by Ssy1 involves an SCF^{Grr1}-catalysed ubiquitination step. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ssy1p; Signalling; Ubiquitin; SCF; AGP1; PTR2

1. Introduction

Among the ~270 yeast proteins classified as membrane transporters [1–3], some are proposed to act as sensors of external compounds. For instance, Snf3 and Rgt2 are similar to hexose facilitators (Hxt proteins) but their main role is to control transcription of *HXT* genes in response to external glucose [4]. Unlike classical Hxt transporters, Snf3 and Rgt2 are expressed at low levels and they harbour a much larger cytosolic C-terminal domain essential to their function as glucose sensors [5]. A central component of the transduction pathway activated by Snf3 and Rgt2 is the SCF^{Grr1} ubiquitin–ligase complex [6]. This complex is best known for its role in ubiquitination and degradation of cyclins Cln1 and Cln2 [7]. In the glucose signalling pathway, SCF^{Grr1} is proposed to modulate, directly or indirectly, the activity of Rgt1, a Cys_6-Zn_2 family transcription factor acting both as a repressor (low glucose) and as an activator (high glucose) of *HXT* gene transcription [8].

Another yeast permease-like sensor, Ssy1, is homologous to amino acid permeases [9–11]. Ssy1 differs from the latter proteins by its much longer N-terminal tail (278 amino acids), two larger extracellular loops connecting transmembrane domains and a lower expression level [12]. Ssy1 is essential to transcriptional induction in response to multiple amino acids of the *AGP1* gene encoding a broad-specificity amino acid permease, of six other amino acid permease genes (*BAP2*, *BAP3, GNP1, BAP2, TAT1* and *TAT2*) and of the di-tri-peptide permease gene *PTR2* [9,10]. The large N-terminal tail of Ssy1, factors Ptr3 and Ssy5 and transcription factor Uga35/Dal81 are essential components of the Ssy1 transduction pathway [10–13]. Furthermore, the F-box protein Grr1, essential for transduction of the glucose signals generated by Snf3 and Rgt2 [6], is also essential for induction by amino acids of the *AGP1* gene [10]. However, little is known about the exact role of Grr1 in the amino acid signalling pathway.

In this paper we show that ubiquitin, other components of the SCF^{Grr1} ubiquitin–ligase complex and the ubiquitin-conjugating enzyme Cdc34 are required for transcriptional induction in response to amino acids of the *AGP1* and *PTR2* genes. This strongly suggests that the ubiquitination of a factor is required for transduction to these genes of the amino acid signal generated by Ssy1.

2. Materials and methods

2.1. Strains, growth conditions and methods

The *Saccharomyces cerevisiae* strains used in this study are all isogenic with the wild-type Σ1278b [14] except for the mutations mentioned (Table 1). Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose or galactose (when mentioned) as the carbon source [15]. To this medium, urea (5 mM), proline (5 mM), $(NH_4)_2SO_4$ (10 mM), amino acids (1–10 mM), or combinations of these compounds were added as a source(s) of nitrogen. Assays for resistance to toxic amino acid analogues were carried out on plates with $(NH_4)_2SO_4$ (10 mM) as the sole nitrogen source. Analogue concentrations were as follows: 20 μg/ml, β-(2-thienyl)-D,L-alanine; and 20 μg/ml, D,L-ethionine. All procedures for manipulating DNA were standard ones [16,17]. The *Escherichia coli* strain used was JM109.

2.2. β-Galactosidase assays

All β-galactosidase assays were performed on cells that reached the state of balanced growth except for cells harvested at various times after the addition of an amino acid in the culture medium. β-Galactosidase assays were measured as described earlier [18] and are expressed as nmol of *o*-nitrophenol formed per min per mg of protein. Protein concentrations were measured with the Folin reagent and bovine serum albumin as the standard [19].

3. Results and discussion

3.1. Components of the SCF^{Grr1} ubiquitin–ligase complex are required for induction of the AGP1 gene

Induction of *AGP1* by amino acids requires Grr1 [10], the F-box protein part of the SCF^{Grr1} ubiquitin–ligase complex

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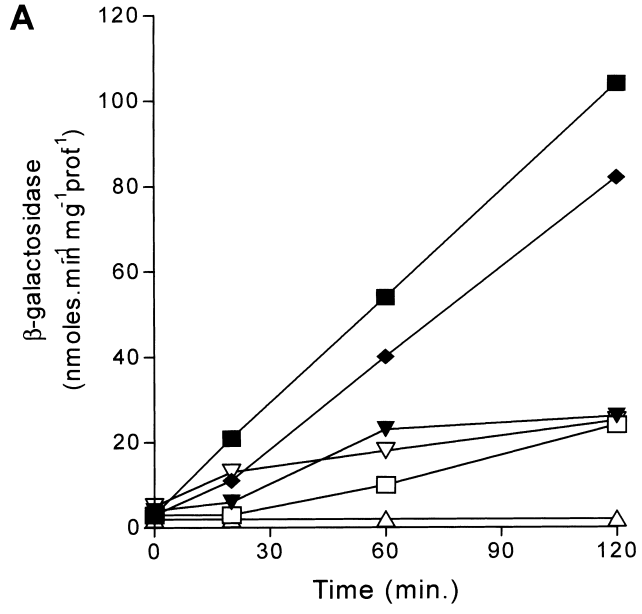
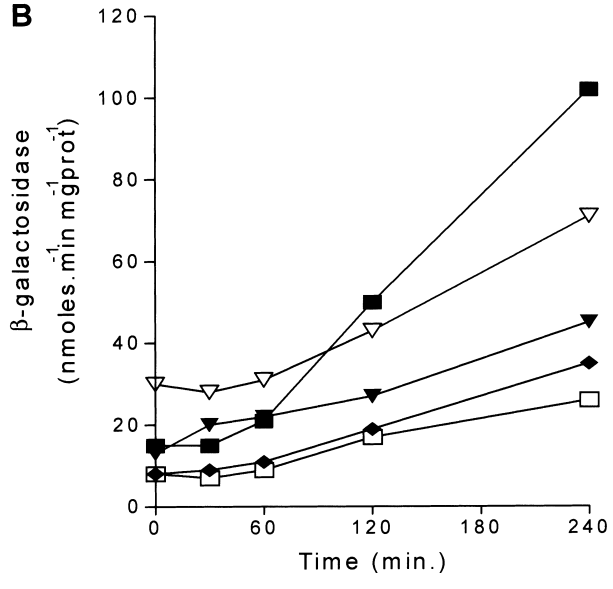
Table 1
Yeast strains used in this study

Strain	Genotype	Reference or source
23344c	*MAT*α *ura3*	M. Grenson
30629c	*MAT*a *gap1*Δ::*kanMX2 ura3*	[10]
32501d	*MAT*a *gap1*Δ::*kanMX2 ssy1*Δ::*kanMX2 ura3*	[10]
34277b	*MAT*a *gap1*Δ::*kanMX2 grr1*Δ::*kanMX2 ura3*	This study
27002d	*MAT*a *npi2 ura3*	[33]
27061b	*MAT*a *trp1*	[25]
27071b	*MAT*a *trp1 npi2*	[25]
JA115	*MAT*α *grr1*Δ::*kanMX2 ura3*	[10]
MT235	*MAT*a *his3-11, leu2-3, ura3-1, ade2-1, trp1-1, can1-100*	M. Tyers
MT670	*MAT*a *his3-11, leu2-3, ura3-1, ade2-1, trp1-1, can1-100, cdc34-1*	[34]
MT871	*MAT*a *his3-11, leu2-3, ura3-1, ade2-1, trp1-1, can1-100, cdc53-1*	[34]
Y554	*MAT*a *his3-11, leu2-3, ura3-1, ade2-1, trp1-1, can1-100, skp1-12*	[35]
HRT1-myc9	*MAT*a *can1-100, leu2-3, -112, his3-11,-15, trp1-1, ura3-1, ade2-1, pep4::TRP1, bar1::LEU2, hrt1::HRT1myc9-HIS5*	[36]





[20]. Other components of SCF^{Grr1} include Cdc53, Skp1 and the Ring-H2 finger protein Hrt1 [20]. More recently, Sgt1 has been identified as another component of SCF^{Grr1} [21]. Cdc34 is the ubiquitin-conjugating enzyme involved in all thus far described SCF-mediated protein degradation processes. To determine if Grr1 is required for *AGP1* expression as a component of the SCF^{Grr1} complex, we tested whether Cdc34 and three other SCF^{Grr1} components besides Grr1 (Cdc53, Skp1, Hrt1) are also required for *AGP1-lacZ* induction (Fig. 1A). As expected, induction of *AGP1-lacZ* was totally impaired in the *grr1*Δ strain. It was also strongly impaired in the *cdc34-1*, *skp1-12* and *hrt1* thermosensitive mutants, suggesting that the SCF^{Grr1} complex plays an important role in *AGP1* induction. Unexpectedly, induction of *AGP1* was almost unaffected in the *cdc53-1* mutant (Fig. 1A). Hence, either Cdc53 is not involved in *AGP1* induction, or the *cdc53-1* mutation does not impair the role of Cdc53 in *AGP1* induction. It is noteworthy that the *cdc53-1* mutation used in this experiment likewise did not affect another function involving all other components of the SCF complex [22]. Other conditional alleles of the *CDC53* gene should thus be tested to assess the role of this protein in *AGP1* induction.

3.2. A normal pool of ubiquitin is essential to induction of *AGP1* expression

The important role of SCF^{Grr1} components and Cdc34 in *AGP1* induction suggests that transduction to this gene of the amino acid signal generated by Ssy1 involves a ubiquitination step. To assess the importance of ubiquitin in the Ssy1 pathway, we monitored expression of the *AGP1* gene in an *npi2/doa4* strain. The *DOA4* gene encodes a ubiquitin hydrolase important for recycling ubiquitin during protein degradation by the proteasome [23]. In the *npi2/doa4* strain used in this

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Fig. 1. Induction of *AGP1* (A) and *PTR2* (B) are impaired in mutants defective in components of the SCF^{Grr1} ubiquitin–ligase complex. Yeast cells transformed with *AGP1::lacZ* or *PTR2::lacZ* reporter plasmid were pregrown at 30°C to mid-log phase on urea minimal medium. Cells were then shifted to 37°C for 60 min before isoleucine 5 mM was added to the media. Cell samples were taken at the indicated time intervals and assayed for β-galactosidase activity. The strains bearing thermosensitive mutations (except for *grr1*Δ) were MT235 (wild type, ■), MT670 (*cdc34-1*, ▼), Y554 (*skp1-12*, □), MT871 (*cdc53-1*, ♦) HRT1 (*HRT1*myc9, ▽) and JA115 (*grr1*Δ, △) transformed by plasmid *YCpLEU2-HIS3* (*pJYS20*), *YEpTRP1* (*pFL45s*) and *YCpAGP1::lacZ*.

Table 2
A normal pool of ubiquitin is required for induced transcription of *AGP1* gene

	β-Galactosidase activity (nmol/min/mg of protein)							
	AGP1-lacZ				*ARO9-lacZ*			
	−Ub		+Ub		−Ub		+Ub	
	–	Phe	–	Phe	–	Phe	–	Phe
Wild type	9	609	12	403	2	955	5	891
npi2/doa4	4	18	12	324	5	261	5	637

Strains 27061b (*trp1, ura3*) and 27071b (*trp1, ura3, npi2*$^{-}$) were transformed by the CEN-based plasmid YCp*AGP1-lacZ* or YCp-*ARO9-lacZ* and pFL39 (−Ub) or YEp96 (+Ub) plasmids. YEp96 contains a synthetic yeast Ub gene under the control of the copper-inducible *CUP1* promoter [37]. Cells were grown on minimal medium with urea as the sole nitrogen source, then $CuSO_4$ (1 mM) was added to the culture to induce overproduction of ubiquitin. After 2 h of induction, phenylalanine was added at 5 mM final concentration. Cells where harvested at time 0 of induction by phenylalanine and after 3 h. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 20%.

study, the internal ubiquitin pool is reduced several-fold, and this leads to impaired ubiquitination of many proteins [24,25]. The results show that induction of *AGP1-lacZ* expression is severely impaired in the *npi2/doa4* mutant (Table 2). To confirm that this effect is due to a reduced ubiquitin pool, we repeated the experiment using *npi2/doa4* strains transformed with a high-copy-number vector bearing the ubiquitin gene (*UBI1*) under the control of the copper-inducible *CUP1* promoter. The results of the β-galactosidase assays show that impairment of induced *AGP1-lacZ* expression in the *npi2/doa4* mutant is indeed largely relieved if the cells overproduce ubiquitin (Table 2). As ubiquitin plays an important role in many cellular functions including transcription, a control experiment using *ARO9-lacZ* as the reporter gene was conducted in parallel. The *ARO9* gene encodes aromatic aminotransferase II, the enzyme catalysing the first step of tryptophan, phenylalanine and tyrosine catabolism. This gene is induced by aromatic amino acids independently of Ssy1 [26]. Whereas phenylalanine-triggered induction of *AGP1* expression was reduced ∼30-fold in the *npi2/doa4* strain, that of *ARO9* was reduced only ∼3-fold, an effect largely compensated by overproduction of ubiquitin (Table 2). It thus seems that ubiquitin plays a much more important role in phenylalanine-triggered induction of *AGP1* expression than in phenylalanine-triggered induction of *ARO9* expression. These results reinforce the view that ubiquitin plays an important role in the Ssy1 transduction pathway.

The SCF complex is modified by attachment of Rub1/Nedd8, a ubiquitin-like protein, to the Cdc53/Cullin subunit [27]. This modification, observed in organisms ranging from yeast to humans, appears to enhance the activity of the SCF complex [27,28]. We monitored expression of the *AGP1-lacZ* gene in *rub1*Δ cells but did not observe any significant change (Table 3). Similarly Smt3, another ubiquitin-related protein, does not seem to play an important role in *AGP1* expression, as judged by the lack of effect of a thermosensitive *smt3* mutation [29] on expression of the *AGP1-lacZ* gene (data not shown).

3.3. F-box protein Grr1p is involved in PTR2 expression and utilisation of various amino acids

Lack of *AGP1* expression in a *gap1*Δ mutant defective in the general amino acid permease leads to very poor growth when one of several amino acids, such as leucine, phenylalanine, or methionine, is supplied at low concentration (1 mM) as the sole nitrogen source. Deletion of the *SSY1* gene in the same *gap1* background has a broader effect on amino acid utilisation because this gene is required for induction of other amino acid permease genes besides *AGP1* [10]. As a first step in investigating whether Grr1 is involved in expression of the other amino acid permease genes under Ssy1 control, we compared the growth phenotype of [*gap1*Δ *agp1*Δ], [*gap1*Δ *ssy1*Δ] and [*gap1*Δ *grr1*Δ] strains (Fig. 2 and data not shown). The [*gap1*Δ *grr1*Δ] mutant grew normally on NH_4^+ medium, but it failed to grow under conditions which affected the growth of the [*gap1*Δ *ssy1*Δ] strain. Furthermore, the [*gap1*Δ *grr1*Δ] strain grew very slowly on a high concentration (10 mM) of methionine, leucine, threonine, or phenylalanine, conditions under which the [*gap1*Δ *ssy1*Δ] strain grew normally or nearly so. Hence, Grr1 seems essential for expression of other amino acid permease genes, in addition to *AGP1*, which are under Ssy1 control. Furthermore, the fact that a Grr1 deficiency has a broader impact spectrum than a Ssy1 deficiency suggests that Grr1 is required for expression of amino acid utilisation genes not under Ssy1 control. We then tested if a gene not involved in amino acid uptake though under Ssy1 control, i.e. the di-tri-peptide permease gene *PTR2*, requires Grr1 for induction by amino acids. The results of experiments monitoring expression of *PTR2-lacZ* indeed showed that *PTR2-lacZ* remains uninduced in the *grr1*Δ strain (Table 3). We also tested if other components of the SCF^{Grr1} complex are involved in induction of *PTR2* (Fig. 1B). We found that induction by tryptophan of *PTR2-lacZ* is impaired in *skp1-12, hrt1* and *cdc34-1* mutant strains. Interestingly, unlike what was observed for *AGP1-lacZ*, induction of *PTR2-lacZ* was also impaired in the *cdc53-1* mutant (Fig. 1B). This suggests that the mechanisms through which Cdc53 within the SCF^{Grr1} complex mediates induction of *AGP1* and *PTR2* are not strictly the same. For instance, the target protein of SCF^{Grr1}

Table 3
The Grr1 protein is required for induced transcription in response to multiple amino acids of *AGP1* and *PTR2* genes

Line no.		β-Galactosidase activity (nmol/min/mg of protein)							
		AGP1-lacZ				*PTR2-lacZ*			
		–	Leu	Met	Trp	–	Leu	Met	Trp
1	wild type	≤2	1036	933	1577	4	35	37	89
2	*grr1*Δ	≤2	≤2	≤2	12	5	4	4	4
3	*rub1*Δ	≤2	980	1181	1657	n.d.	n.d.	n.d.	n.d.

Strains 23344c (*ura3*), JA115 (*grr1*Δ) and JA170 (*rub1*Δ) transformed by the CEN-based plasmid YCp*AGP1-lacZ* or YCp*PTR2-lacZ* plasmid were grown on minimal urea medium with the added indicated compounds at 5 mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 20%. n.d.: not determined.

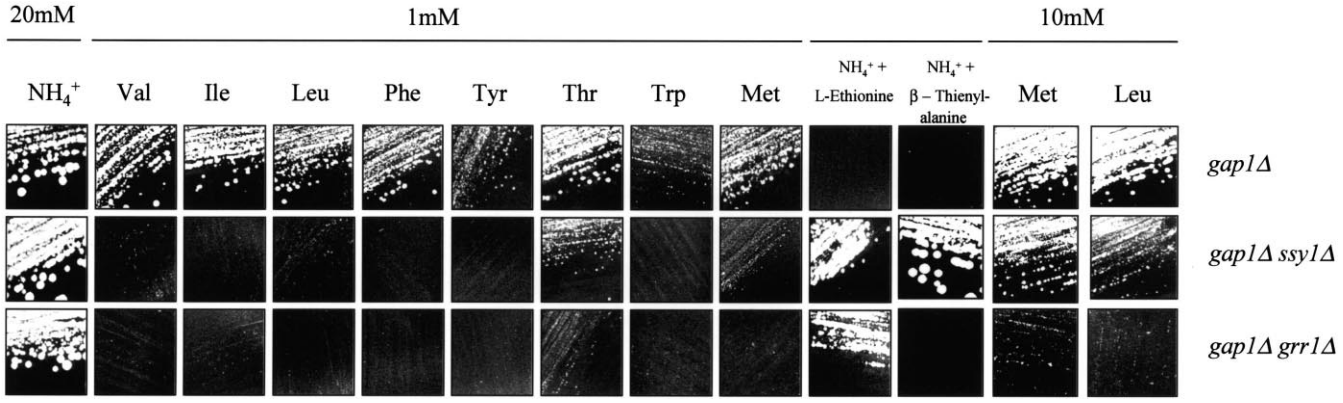

Fig. 2. Deletion of the *GRR1* gene affects the utilisation of several amino acids in cells lacking the general amino acid permease (Gap1p). Cells were spread on minimal medium with the indicated nitrogen source at the indicated concentration. The amino acid toxic analogues β-thienyl-alanine and L-ethionine were added at a final concentration of 20 μg/ml. The strains were 30629c (*gap1Δ ura3*), 32501d (*gap1Δ ssy1Δ ura3*) and 34277b (*gap1Δ grr1Δ ura3*).

in induction of each gene could be different. Alternatively, SCF^{Grr1} could act in the context of gene promoters [30] whose constraints for gene activation might be different for both genes. A striking feature of *PTR2* expression is that it is regulated by two different ubiquitin ligases, namely Ubr1 and the SCF^{Grr1} complex. The first is involved in the intracellular peptide induction pathway (Ubr1/Cup9) [31] and the second in the extracellular amino acid induction pathway (Ssy1/Ptr3/Ssy5/SCF^{Grr1}) (Fig. 1B).

In conclusion, these results support the idea that SCF^{Grr1} complex and ubiquitin play an important role in the external amino acid signalling pathway. However, their precise role remains to be determined. Possibly, expression of Ssy1 target genes is prevented when some factor is not degraded via the SCF^{Grr1} ubiquitin pathway. The factor might be a negative component of the signalling pathway, or a positive component that needs to undergo ubiquitin-dependent processing to be activated in response to amino acids. Another possibility is that the Ssy1 pathway does not include any ubiquitination step and that the factor accumulating in ubiquitin hydrolase or SCF components mutants is part of another transcriptional control mechanism acting in a dominant negative fashion on genes controlled by Ssy1p. This would imply that induction of Ssy1p-regulated genes by external amino acids depends both on activation of the Ssy1 pathway and on SCF^{Grr1}-dependent relief from a negative control mechanism in response to unknown signals. The genetic analysis of suppressors of *grr1* mutations should help to unravel the exact roles of SCF^{Grr1} and ubiquitin in transcription of amino acid and peptide permease genes.

Remarkably, the components of the SCF^{Grr1} complex are also required for expression of the *HXT* genes regulated by the Snf3 and Rgt2 glucose sensors [6]. Since a mutation in *CDC34* was found not to affect induction of the *HXT* genes, it could be that another ubiquitin-conjugating enzyme is involved [8]. As in the Ssy1 pathway, the protein targets of SCF^{Grr1} pathway in the glucose signalling remain unknown [6]. The SCF^{Grr1} complex is best known for its role in the degradation of cyclins Cln1 and Cln2 [7,28] and of Gic1 and Gic2, two proteins regulating actin polymerisation and bud emergence [32]. An unanswered key question is whether the role of SCF^{Grr1} in control of glucose and amino acid transport is coupled to its involvement in cell cycle regulation.

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