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Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle

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Grr1 protein of the yeast *Saccharomyces cerevisiae* is a central component of a glucose signal transduction mechanism responsible for glucose-induced gene expression. It is required for glucose-stimulated regulation of Rgt1, a repressor of several glucose-induced *HXT* genes. Grr1 also plays a role in regulating the cell cycle, because it is required for degradation of the G_1 cyclins Cln1 and Cln2. We discovered that Grr1 physically interacts with Skp1, a protein that has been implicated in a ubiquitin-conjugating enzyme complex that targets for degradation the cell cycle regulators Cln1 and Cln2, and the cyclin-dependent kinase inhibitor Sic1. Thus, Grr1 may regulate the cell cycle and glucose-induced gene expression via ubiquitin-mediated protein degradation. Consistent with this idea, Skp1, like Grr1, was found to be required for glucose-induced *HXT* gene expression. Two functional domains of Grr1 are required for its interaction with Skp1: 12 leucine-rich repeats (LRR) and an adjacent F-box. The Grr1–Skp1 interaction is enhanced by high levels of glucose. This could provide yeast with a mechanism for coupling nutrient availability to gene expression and cell cycle regulation.

Keywords: glucose signaling/G_1 cyclin turnover/leucine-rich repeats/*Saccharomyces cerevisiae*

Introduction

Glucose, the preferred carbon source for the yeast *Saccharomyces cerevisiae*, induces transcription of several genes necessary for its metabolism and represses transcription of many genes required for its oxidation and for utilization of other carbon sources (reviewed by Trumbly, 1992; Johnston and Carlson, 1993; Ronne, 1995). Transcriptional regulation by glucose requires the transduction of a glucose signal to transcriptional regulatory proteins, and the Grr1 protein appears to play a central role in this process: mutations in *GRR1* relieve repression of many glucose-repressed genes (Bailey and Woodword, 1984; Flick and Johnston, 1991) and prevent glucose induction of several *HXT* genes encoding glucose transporters (Özcan and Johnston, 1995). Genetic analysis suggested that *GRR1* acts at an early stage of glucose signal transduction to inhibit the function of Rgt1, a transcriptional repressor of the *HXT* genes, thereby causing derepression of *HXT* gene expression (Flick and Johnston, 1991; Erickson and Johnston, 1994; Vallier *et al*., 1994; Özcan and Johnston, 1995; Özcan *et al*., 1996).

In addition to defective glucose signaling, *grr1* mutants display a number of other defects, including elongated cell morphology and reduction in transport of aromatic amino acids and divalent cations (Bailey and Woodword, 1984; Flick and Johnston, 1991; Conklin *et al*., 1993), suggesting that Grr1 performs functions in addition to regulating Rgt1. Indeed, *GRR1* is required for degradation of the G_1 cyclins Cln1 and Cln2 (Barral *et al*., 1995), the proteins that catalyze progression through the start point of the cell cycle by activating the Cdc28 cyclin-dependent kinase. This observation raises the possibility that Grr1 is directly involved in the process of protein degradation. Perhaps Grr1 acts in different cellular pathways (i.e. glucose induction and G_1 cyclin turnover) by targeting various regulatory proteins such as Rgt1 or Cln1 for degradation.

The G_1 cyclins are very unstable proteins (Wittenberg *et al*., 1990; Tyers *et al*., 1992; Salama *et al*., 1994; Lanker *et al*., 1996) that are thought to be degraded via the ubiquitin proteolysis pathway, since both Cln2 and Cln3 are ubiquitinated and degraded in a Cdc34-dependent manner (Tyers *et al*., 1992; Deshaies *et al*., 1995; Yaglom *et al*., 1995). Cdc34 is a ubiquitin-conjugating enzyme (E2) that, in combination with a ubiquitin–protein ligase (E3), adds ubiquitin to substrate proteins (e.g. Cln1, Cln2), thereby targeting them for degradation by the 26S proteasome (Goebl *et al*., 1988; reviewed by Hochstrasser, 1995; King *et al*., 1996). In addition to *CDC34*, *CDC53* (Willems *et al*., 1996), *SKP1* (Bai *et al*., 1996) and *GRR1* (Barral *et al*., 1995) are required for G_1 cyclin degradation. Cdc53, Skp1 and Grr1 are therefore candidates for E3 components of the ubiquitin-conjugating enzyme for G_1 cyclin degradation (reviewed by Jackson, 1996; King *et al*., 1996). Consistent with this model, Cdc53 was shown to associate with Cdc34 and with the unstable form of Cln2 (Willems *et al*., 1996), and it acts in concert with Cdc4 and Cdc34 to control the G_1–S phase transition of the cell cycle (Mathias *et al*., 1996).

Skp1 is a yeast protein that connects cell cycle regulators to the ubiquitin proteolysis machinery through its interaction with components of a ubiquitin-conjugating enzyme complex, including Cdc4 (Bai *et al*., 1996) and Cdc53 (E.Patton and M.Tyers, personal communication). *SKP1* is required for ubiquitin-mediated proteolysis of Cln2, Clb5 and the cyclin-dependent kinase (Cdc28) inhibitor Sic1 (Bai *et al*., 1996). Skp1 and Cdc4 directly interact through a sequence motif in Cdc4 called the F-box, which is also found in Grr1 (Bai *et al*., 1996). The human homolog of Skp1 (for which the protein is named: S-phase kinase-associated protein), is present in a protein complex with cyclin A–CDK2 and Skp2, a protein essential for G_1–S phase cell cycle progression (Zhang *et al*.,

1995). Skp1 appears to participate in diverse cellular functions because it is also a subunit of the centromere-binding complex CBF3 (Connelly and Hieter, 1996).

Grr1 appears to be organized similarly to Cdc4 and human Skp2. In addition to their F-boxes, which have been proposed to be Skp1 interaction domains (Bai *et al*., 1996), all three proteins possess an additional potential protein–protein interaction domain: Grr1 contains 12 leucine-rich repeats (Flick and Johnston, 1991; Kobe and Deisenhofer, 1994), Skp2 contains seven leucine-rich repeats (but of a family different from the Grr1 repeats; Zhang *et al*., 1995), and Cdc4 contains seven WD40 repeats (Yochem and Byers, 1987; Neer *et al*., 1994). Because of its apparent similarity to these two Skp1-interacting proteins, it has been proposed that Grr1 also interacts with Skp1 (Bai *et al*., 1996). We report here that this is indeed the case. This observation, together with the finding that Skp1, like Grr1, is required for glucose-induced *HXT* gene expression, leads us to propose that Grr1 acts with Skp1 in a ubiquitin-conjugating enzyme complex to target various proteins (Cln1, Cln2 and perhaps Rgt1) for degradation. The Grr1–Skp1 interaction is enhanced by high levels of glucose, which could provide yeast with a mechanism to couple availability of one of its most important nutrients—glucose—to gene expression and cell cycle progression.

Results

Isolation of Skp1 as a Grr1-interacting protein

Because the 12 leucine-rich repeats that Grr1 contains are a likely protein–protein interaction domain, we sought by the two-hybrid method to identify proteins that interact with Grr1 (Fields and Song, 1989). We constructed two Grr1 'baits' by fusing it to either the LexA or Gal4 DNA-binding domains (LexA–Grr1 or BD–Grr1 respectively; Figure 1A). The fragment of Grr1 used in the fusion, Grr1-ΔN, is missing the N-terminal 280 amino acid residues, and appears fully functional because it complements a *grr1* mutant (Figure 1B).

Using LexA–Grr1 as bait, we screened a yeast two-hybrid cDNA library and isolated a clone that showed specific interaction with Grr1 (Figure 1C). The DNA sequence of the cDNA insert revealed that it encodes Skp1 (Bai *et al*., 1996; Connelly and Hieter, 1996), beginning at amino acid 13. In another two-hybrid screen using BD–Grr1 as bait, we identified another Grr1-interacting clone containing *SKP1* fused to Gal4AD beginning at the 11th codon of *SKP1* (Figure 1A). Each *SKP1* clone interacts specifically with both BD– and LexA–Grr1 bait proteins (Figure 1C).

The Grr1–Skp1 interaction was confirmed *in vivo* by co-immunoprecipitation. Yeast extracts were prepared from glucose-grown cells expressing both hemagglutinin epitope-tagged Grr1 (Grr1-$\Delta N^{BD\text{-}HA}$) and $Skp1^{AD}$. When Grr1-$\Delta N^{BD\text{-}HA}$ was immunoprecipitated with anti-HA antibody, $Skp1^{AD}$ was detected only in immunoprecipitates prepared from cells expressing both fusion proteins (Figure 2A, lanes 2–5). In the converse experiment, $Grr1^{BD\text{-}HA}$ was found only in anti-AD immunoprecipitates from extracts containing both fusion proteins (Figure 2B, lanes 2–5). These results confirm that Grr1 associates with Skp1 in glucose-grown cells.

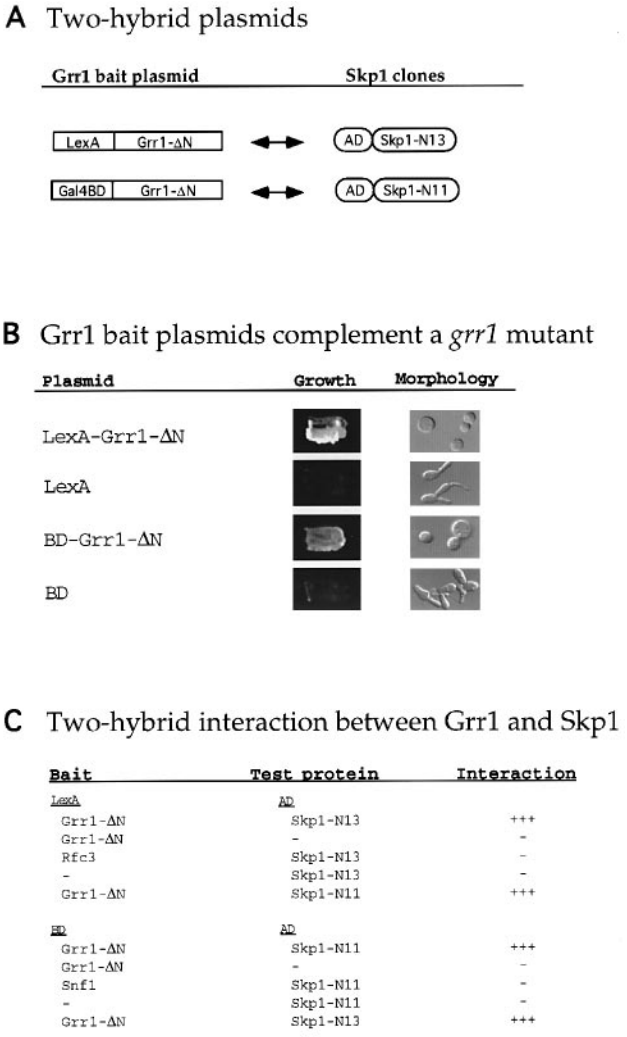

Fig. 1. Isolation of Skp1 as a Grr1-interacting protein. (**A**) Schematic representation of the Grr1 bait plasmids used in the two-hybrid screens and the Skp1 clones isolated. (**B**) Growth on YPD and cellular morphology of a *grr1* mutant carrying the indicated plasmids. (**C**) Two-hybrid interaction between indicated clones as monitored by the β-galactosidase filter lift assay of patches of cells. '+++' indicates that cells developed blue color within 30 min of assay. '–' indicates cells remain white over the period of the assay (2–4 h).

Grr1 interacts with Skp1 in vitro

The Grr1–Skp1 interaction was tested *in vitro*. GST–Skp1 protein was produced in *Escherichia coli* and immobilized on glutathione–agarose beads. ^{35}S-labeled Grr1 made by *in vitro* translation (see Materials and methods) was then incubated with GST–Skp1. Grr1 (missing its 280 N-terminal amino acids, Grr1-ΔN) binds more efficiently to GST–Skp1 than to GST alone (Figure 3, lanes 4–5). Further removal of the C-terminal 230 amino acids of Grr1 (Grr1-ΔNC) did not affect the interaction (Figure 3, lanes 6–7). This suggests that Grr1 can directly interact with Skp1, and neither its N- nor C-terminal regions are required for interaction. The F-box of Grr1 is required for interaction with Skp1 (Figure 3, lanes 8–9, see below).

The F-box and leucine-rich repeats of Grr1 are required for interaction with Skp1

Grr1 contains two important domains: the F-box and leucine-rich repeats (Figure 4). Since the F-box was

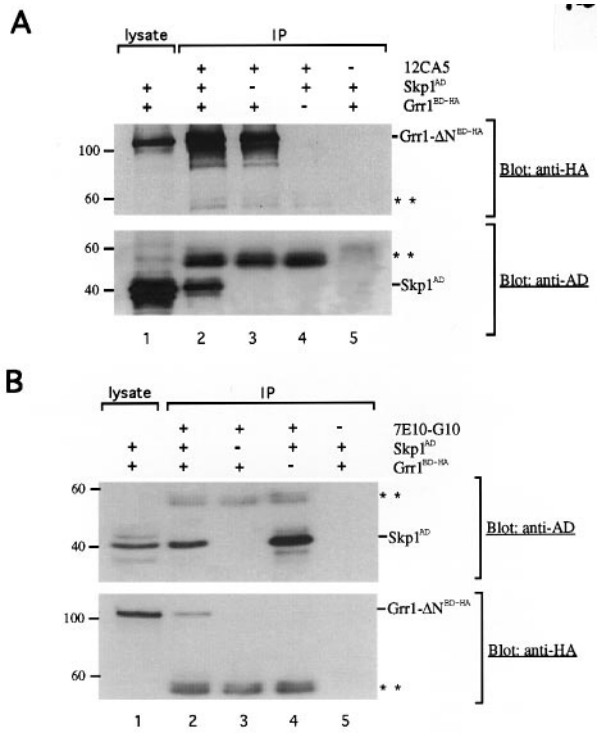

Fig. 2. Grr1p interacts with Skp1p *in vivo*.
(**A**) Co-immunoprecipitation of Skp1 and Grr1 using HA antibody (12CA5). Lysates from strains expressing the indicated tagged Grr1 and Skp1 proteins from the *ADH1* promoter were immunoprecipitated with anti-HA antibody (lanes 2–4) or no antibody (lane 5). (We are unable to detect Grr1 expressed from its own promoter.) The immunoprecipitate was resolved by SDS–PAGE, blotted and probed with HA antibody (top panel) and Gal4AD antibody (bottom panel). (**B**) Co-immunoprecipitation of Grr1 and Skp1 using Gal4AD antibody (7E10-G10). Lysates from strains expressing the indicated tagged Grr1 and Skp1 proteins were immunoprecipitated with anti-Gal4AD antibody (lanes 2–4) or no antibody (lane 5). The immunoprecipitate was resolved by SDS–PAGE, blotted and probed with Gal4AD antibody (top panel) and HA antibody (bottom panel). Double asterisks indicate the positions of the immunoglobulin heavy chains. The positions of Grr1 and Skp1 proteins are indicated. The molecular mass standards (left) are given in kDa.

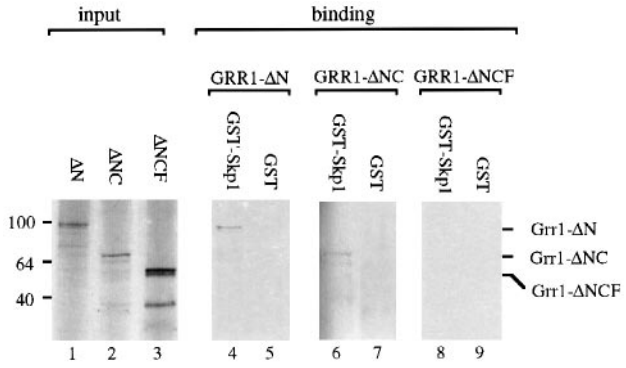

Fig. 3. *In vitro* binding assay of Grr1 and Skp1. The indicated Grr1 proteins (the following amino acids were deleted: N = 280 N-terminal; C = 230 C-terminal; F = 280–390, F-box region) were expressed and labeled with [^{35}S]cysteine by *in vitro* translation (as shown in lanes 1–3) and incubated with GST–Skp1 or GST protein immobilized on glutathione–agarose beads. Grr1 bound to the beads was detected by SDS–PAGE and fluorography. The panels showing the binding results are all exposed 5-fold longer than the one showing the input. The binding assays used 2.5-fold the amount of *in vitro*-labeled Grr1 as compared with the input lanes. The positions of Grr1 proteins are marked. The molecular mass standards (left) are given in kDa.

identified as the Skp1-interacting domain in three different proteins, namely Skp2 (Zhang *et al*., 1995), cyclin F and Cdc4 (Bai *et al*., 1996), it is likely that it also mediates interaction between Grr1 and Skp1. The leucine-rich repeats of Grr1 are a likely protein interaction domain (Kobe and Deisenhofer, 1994) that may contribute to Skp1 interaction. Indeed, the F-box of Grr1 is required for its interaction with Skp1 *in vivo* (Figure 4, line 2) and *in vitro* (Figure 3, lane 8), and the leucine-rich repeats are required for interaction with Skp1 *in vivo* (Figure 4, line 3). [The inability of Grr1-ΔNCF and Grr1-ΔNL to interact with Skp1 in the two-hybrid assays cannot be due to reduced protein levels since both proteins are even more abundant than Grr1-ΔN (Figure 4, right panel).] These two domains of Grr1 are sufficient for its interaction with Skp1 because Grr1-ΔNC, which contains little more than the F-box and leucine-rich repeats, interacts with Skp1 *in vitro* (Figure 3, lanes 6–7) and *in vivo* (Figure 4, line 4). These two domains of Grr1 are also sufficient for its function *in vivo*, because *GRR1*-ΔNC complements a *grr1* mutant (our unpublished results). Thus, the F-box and leucine-rich repeats are each necessary and together sufficient for Grr1 function and interaction with Skp1.

Regulation of Grr1–Skp1 interaction by glucose

To address the question of whether Grr1 function is regulated by glucose, we tested whether the interaction between Grr1 and Skp1 is modulated by glucose. The β-galactosidase activity resulting from interaction of Grr1-ΔN and Skp1 in the two-hybrid assay is ~4-fold higher in cells grown on glucose than in cells grown on raffinose (Figure 4, line 1). [The lower level of β-galactosidase activities in raffinose-grown cells is not likely to be due to lower levels of the fusion proteins because Western blots show that their cumulative levels are about the same in both glucose- and raffinose-grown cells (Figure 5A, top and bottom panels, lanes 1 and 2).] Furthermore, much less Skp1^{AD} co-immunoprecipitates with Grr1-ΔN^{BD-HA} from extracts of raffinose-grown cells than from extracts of glucose-grown cells (Figure 5A, bottom panel, lane 3 versus lane 4), even though similar amounts of Grr1 (Figure 5A, top panel, lanes 1–2) and Skp1 (Figure 5A, bottom panel, lanes 1–2) proteins are present in these extracts. Thus, the interaction between Grr1 and Skp1 appears to be enhanced by glucose.

While examining the glucose-mediated regulation of the Grr1–Skp1 interaction by the two-hybrid assay, we were surprised to find that Grr1-ΔNC, which is missing the C-terminal 250 amino acids of Grr1, interacts with Skp1 in raffinose-grown cells almost as well as in glucose-grown cells (Figure 4, line 4). We followed up this observation with an immunoprecipitation experiment. Indeed, Skp1^{AD} was present in about equal amounts in immunoprecipitates of Grr1-ΔNC^{BD-HA} from extracts of glucose- or raffinose-grown cells (Figure 5B, lanes 2 and 4), in contrast to the result with Grr1 carrying an intact C-terminus (Figure 5A, lanes 3 and 4). (Note that deletion of the Grr1 C-terminus appears to reduce the amount of Grr1 in the cell, and thus the total amount of Skp1 that immunoprecipitates with it; see also Discussion.) Thus, the C-terminus of Grr1 may negatively regulate its ability to interact with Skp1 in the absence of glucose.

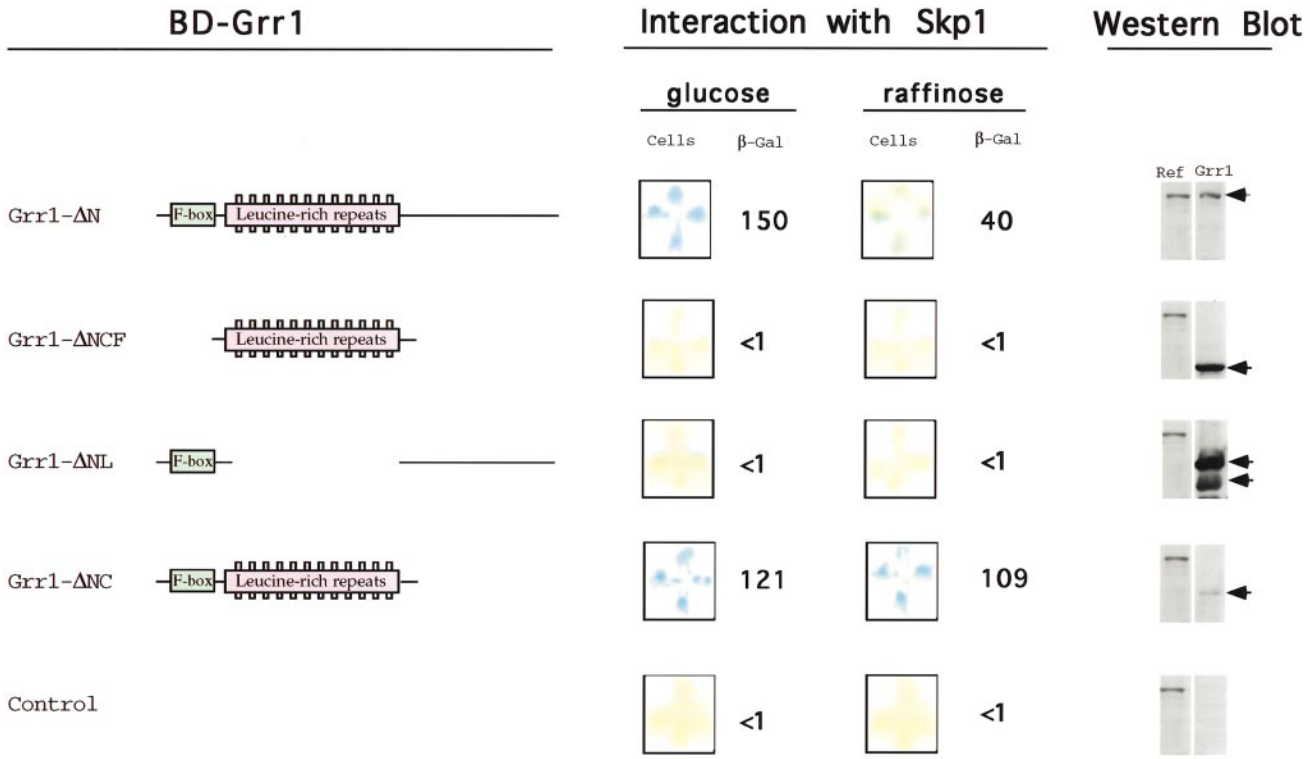

Fig. 4. Mapping the Skp1-interacting domains of Grr1. The interactions between various Grr1 proteins and Skp1 were measured as β-galactosidase activities in the two-hybrid reporter strains (Y190) co-expressing the indicated BD-Grr1 and AD-Skp1 growing on either glucose (left column) or raffinose (right column) media. The colored panels show the result of a filter lift assay for β-galactosidase activity; the adjacent number is the average β-galactosidase activity in three transformants (standard deviations of all assays were <15%). All Grr1 proteins tested are deleted for the N-terminal 282 amino acids (ΔN). The C-terminal region deleted (ΔC) includes amino acids 900–1150. The F-box region (F) includes amino acids 320–370. The 12 leucine-rich repeats (L) span amino acids 410–725. $Grr1^{BD\text{-}HA}$ proteins were examined from glucose-grown yeast extracts by Western blot using HA antibody (right column; positions marked with arrow). Grr1-$\Delta N^{BD\text{-}HA}$ was included as a reference (Ref).

GRR1 overexpression exacerbates the growth defect of skp1-11

To evaluate the significance of Grr1–Skp1 complexes *in vivo*, we examined genetic interactions between *GRR1* and *skp1*. It has been previously shown that *CDC4* overexpression suppresses the *skp1-11* mutation (Bai *et al*., 1996). In contrast, we found that overexpression of *GRR1* exacerbated the growth defect of *skp1-11* cells at permissive temperatures. This was first observed as an inability to transform a *skp1-11* temperature-sensitive mutant (at permissive temperature) with a high-copy *GRR1* plasmid. This was verified by a plasmid loss experiment, in which *skp1-11* cells were co-transformed with a high-copy *GRR1–LEU2* plasmid and a *SKP1–URA3* plasmid. Cells carrying these two plasmids cannot grow on medium lacking leucine (selects for the high-copy *GRR1* plasmid) and containing 5-FOA (selects for loss of the *SKP1* plasmid) even at temperatures permissive for the *skp1-11* mutation (Figure 6). We imagine that this is due to Grr1 titrating the reduced level of functional Skp1 in the mutant, preventing it from performing its essential function in Sic1 degradation. In any case, these results suggest that Grr1 specifically interferes with an essential function of *skp1-11* (see Discussion). Interestingly, Grr1 lacking its C-terminus more severely exacerbates the *skp1-11* phenotype, since it prevents *SKP1* plasmid loss even at 20°C. Perhaps this is due to its enhanced ability to interact with Skp1 (Figures 4, line 4 and 5B, lane 4).

SKP1 is required for glucose induction of HXT gene expression

The observation that Grr1 and Skp1 interact leads to the prediction that Skp1 is required for glucose induction of *HXT* expression, a process that requires Grr1 (Özcan and Johnston, 1995). This is indeed the case: glucose-induction of *HXT1* expression is severely reduced in the *skp1-11* cells, and abolished in the *skp1-12* cells at both restrictive (Figure 7) and permissive (Table I) temperatures for these mutants. Thus, like *GRR1*, *SKP1* is also required for glucose induction of *HXT1* gene expression. Cdc53, which is also a part of the ubiquitin-conjugating enzyme complex (Willems *et al*., 1996), is also required for glucose induction of *HXT1* expression (Figure 7). Interestingly, *CDC34* is not required for glucose induction of *HXT1* expression, suggesting that a different ubiquitin-conjugating enzyme may act with Grr1 and Skp1 to regulate Rgt1 function (see Discussion).

Discussion

Grr1 is a central component of a glucose signal transduction mechanism that inactivates Rgt1, a repressor of glucose-induced genes. Grr1 also plays a role in progression of the cell cycle by regulating turnover of G_1 cyclins. We have presented genetic and biochemical evidence that Grr1 interacts physically with Skp1, a protein known to be involved in ubiquitin-mediated proteolysis of cell cycle

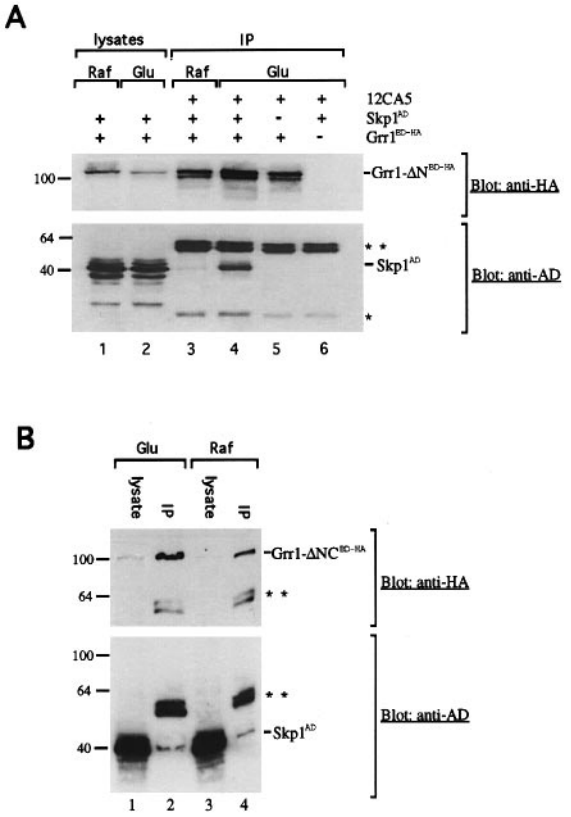

Fig. 5. Regulation of Grr1–Skp1 interaction by glucose. (**A**) Grr1–Skp1 interaction is enhanced by high level of glucose. Lysates from raffinose-grown (lanes 1 and 3) or glucose-grown (lanes 2, 4–6) yeast cells expressing epitope-tagged Grr1-$\Delta N^{BD\text{-}HA}$ and/or $Skp1^{AD}$ were immunoprecipitated with HA antibody (lanes 3–6), resolved on SDS–PAGE, blotted and probed with HA antibody (top panel) and Gal4AD antibody (bottom panel). (**B**) Glucose regulation of Grr1–Skp1 interaction requires the C-terminal domain of Grr1. Lysates from a strain expressing both epitope-tagged Grr1-$\Delta NC^{BD\text{-}HA}$ and $Skp1^{AD}$ grown on either glucose (lanes 1–2) or raffinose (lanes 3–4) were immunoprecipitated with HA antibody (lanes 2 and 4). The immunoprecipitates were resolved on SDS–PAGE and examined for the presence of Grr1 and Skp1 by immunoblotting with HA antibody (top panel) and Gal4AD antibody (bottom panel). Double and single asterisks indicate the positions of the immunoglobulin heavy and light chains respectively. The positions of Grr1 and Skp1 proteins are indicated. The molecular mass standards (left) are given in kDa.

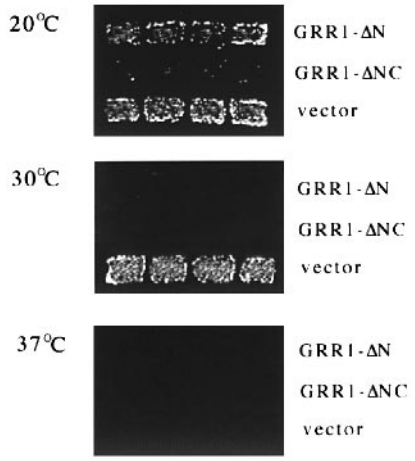

Fig. 6. Overexpression of Grr1 exacerbates the growth defect of *skp1-11* mutant cells. *GRR1–LEU2* overexpression plasmid pBM2868 (*pADH–Grr1-*ΔN^{AD}), pBM3003 (*pADH–Grr1-*ΔNC^{AD}) and control vector pACTII (*pADH–AD*) were co-transformed with pBM3490 (*SKP1–URA3*) plasmid into the *skp1-11* mutant. Transformants were patched and grown on –leu –ura plates for 2 days, stamped onto plates containing 5-FOA and lacking leucine, and allowed to grow at the indicated temperatures for 4 days before being photographed.

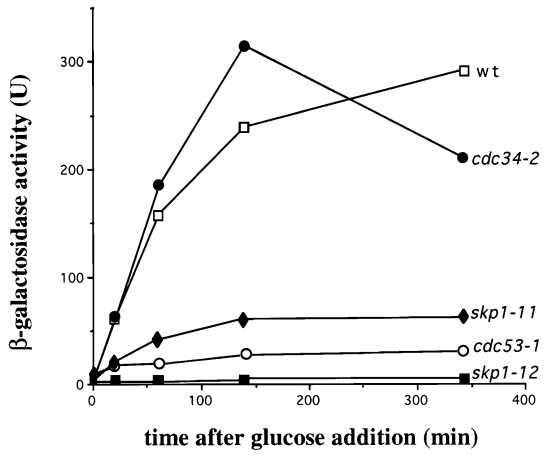

Fig. 7. Mutants of *skp1* are defective in glucose-induced *HXT1* expression at restrictive temperature. Yeast cells transformed with *HXT1::lacZ* reporter plasmid (pBM2636; Özcan and Johnston, 1995) were pregrown at 30°C to mid-log phase on YNB–2% galactose without uracil. Cells were then shifted to 37°C for 60 min before glucose (4%, w/v) was added to the media. Cultures were taken at the indicated time point and assayed for β-galactosidase activity. Standard deviation for each plotted activity is <15%.

regulators (Bai *et al*., 1996). Thus, Grr1 appears to regulate gene expression and cell cycle progression via the ubiquitin proteolysis machinery.

Grr1 is connected to the ubiquitin proteolysis machinery through Skp1

Several lines of evidence suggest that Skp1 is a component of a ubiquitin-conjugating enzyme complex. Skp1, along with Cdc4, Cdc53 and the ubiquitin-conjugating enzyme Cdc34, is required for ubiquitin-mediated proteolysis of Sic1, an inhibitor of the Cdc28 cyclin-dependent protein kinase (Bai *et al*., 1996). Cdc4, Cdc53 and Cdc34 are in a complex (Mathias *et al*., 1996), and Cdc53 has been shown to interact with Cdc34 (Willems *et al*., 1996). Skp1 is probably part of this protein complex because it interacts with Cdc4 (Bai *et al*., 1996). Skp1 was recently shown to be required for interaction between Cdc4 and Cdc53 (E.Patton and M.Tyers, personal communication), supporting the view that Skp1 is an essential component of the Cdc4–Cdc53–Cdc34 complex. Also, Cdc53, Cdc4 and Skp1 (expressed in insect cells) are necessary and sufficient to ubiquitinate Sic1 if supplemented with Cdc34, ubiquitin and ubiquitin-activating enzyme (E1) (R.Feldman, C.Correll and R.J.Deshaies, manuscript in preparation; W.Harper and S.Elledge, unpublished results), suggesting that these three proteins function jointly as an E3, a factor that facilitates the transfer of ubiquitin to the substrate (Sic1) by the ubiquitin-conjugating enzyme (E2). Therefore, Skp1 appears to be an essential component of a ubiquitin-conjugating enzyme complex that may target Sic1 for degradation (Figure 8).

Skp1, along with Cdc53, has also been implicated in a ubiquitin-conjugating enzyme complex that targets G_1 cyclins for proteolysis (Bai *et al*., 1996; Willems *et al*.,

Table I. GRR1 and SKP1 are both required for glucose-induced HXT1 expression

Relevant genotype	Transformed plasmid	Mean β-galactosidase activity (U) ± SD[a]	
		gal[b]	glu[b]
Wild-type		4 ± 1	302 ± 18
grr1	vector[c]	2 ± 0	6 ± 5
grr1	*GRR1*	4 ± 1	290 ± 30
skp1-11	vector	4 ± 2	129 ± 14
skp1-11	*SKP1*	4 ± 3	250 ±35
skp1-12	vector	4 ± 1	7 ± 2
skp1-12	*SKP1*	2 ± 0	273 ± 16

[a]β-Galactosidase activities were all measured from cells grown at 30°C, the permissive temperature for the *skp* mutants.
[b]gal, 2% galactose; glu, 4% glucose.
[c]vector, Yep24.

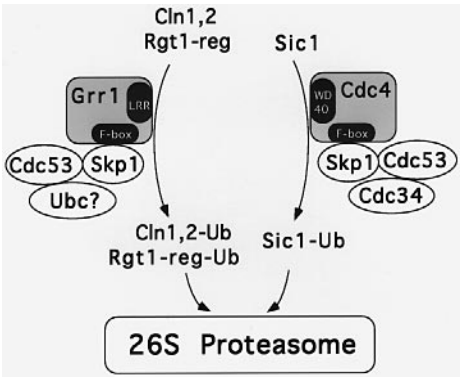

Fig. 8. Model for Grr1 function. Grr1 and Cdc4 are counterparts in different Skp1-containing complexes that target different proteins for ubiquitination. The leucine-rich repeats of Grr1 and WD40 repeats of Cdc4 may function as substrate-recruiting domains; the F-box may mediate interaction with Skp1. Rgt1-reg indicates Rgt1 (or, more likely, its regulator) (see details in Discussion).

1996), a process that requires Grr1 (Barral *et al*., 1995). Since Grr1 interacts with Skp1 and is required for G_1 cyclin degradation and inhibition of Rgt1 function, we propose that Grr1 functions as part of a ubiquitin-conjugating enzyme complex to target G_1 cyclins and Rgt1 (or a regulator of Rgt1; see further discussion below) for degradation (Figure 8). This hypothesis predicts that Skp1 is also required for glucose induction of *HXT* gene expression, and this was indeed found to be the case (Figure 7 and Table I). *SKP1* was not previously identified as a component of the glucose induction mechanism from our genetic analysis, probably because it is an essential gene.

Grr1p contains two important domains. The leucine-rich repeats are a likely protein–protein interaction domain (Kobe and Deisenhofer, 1994) that may serve as a substrate-recruiting domain. Deletion analysis of *GRR1* and identification of additional Grr1-interacting proteins are consistent with the importance of the leucine-rich-repeats in this respect (our unpublished results). The F-box, a Skp1-interacting domain first identified in Cdc4 and cyclin F (Bai *et al*., 1996) and found in several other proteins, including Grr1, may serve to connect Grr1 and its associated targets to the ubiquitin proteolysis machinery (Bai *et al*., 1996). The F-box and leucine-rich repeats are both necessary and sufficient for Grr1 function (our unpublished results) and for its interaction with Skp1 (Figure 4).

Bai *et al*. (1996) proposed that Grr1 and Cdc4 are counterparts in different Skp1-containing complexes based on the fact that they both contain the same Skp1-interacting motif (the F-box) but different probable protein–protein interaction domains (leucine-rich repeats and WD40 repeats, respectively). In fact, Grr1 and Cdc4 appear to have different functions, because Cdc4, but not Grr1, is required for Sic1 degradation: mutants of *cdc4* arrest before S-phase due to their inability to degrade Sic1 (Schwob *et al*., 1994; see also review by King *et al*., 1996), whereas *grr1* mutants proceed rapidly into S phase. Grr1, on the other hand, is required for rapid turnover of G_1 cyclins (Barral *et al*., 1995), but Cdc4 may not be involved in this process, since Cln1 and Cln2 are degraded normally in a *cdc4 sic1* mutant (Blondel and Mann, 1996). We suggest that Cdc4 combines with Skp1, Cdc53 and Cdc34 to ubiquitinate Sic1, and Grr1 combines with the same three proteins (or perhaps with a different ubiquitin-conjugating enzyme; see further discussion below) to ubiquitinate G_1 cyclins and Rgt1 (or its regulator) (Figure 8). Perhaps the WD40 repeats of Cdc4 recruit one set of proteins (Sic1, and possibly others), and the leucine-rich repeats in Grr1 recruit different proteins (G_1 cyclins, Rgt1 and probably others) to the ubiquitin-conjugating enzyme complex.

The pleiotropic defects associated with *grr1* mutants support the view that Grr1 recruits diverse targets to the ubiquitin-conjugating enzyme complex. Mutants of *grr1* have abnormally elongated cell morphology due to G_1 cyclin over-accumulation, and a glucose signaling defect due to uninhibited Rgt1 repressor activity (Özcan *et al*., 1996). The defective aromatic amino acid and cation transport (Flick and Johnston, 1991; Conklin *et al*., 1993) of *grr1* mutants, their sensitivity to nitrogen starvation (Flick and Johnston, 1991) and the synthetic lethality of *grr1* with *cdc55* mutation (Kim *et al*., 1994) probably reflect increased levels of other, unidentified Grr1 targets.

Grr1 and Cdc4 may compete with each other for binding to Skp1. Our observation that Grr1 overexpression exacerbates the growth defect of the conditional *skp1-11* mutant (Figure 6), which is defective in Sic1 degradation (Bai *et al*., 1996), is consistent with this possibility. We imagine that this is due to Grr1 sequestering the limiting amount of Skp1 in this mutant, preventing it from interacting with Cdc4 and thereby preventing degradation of Sic1, which must occur for the cell cycle to progress past the G_1 phase. The behavior of the *skp1-11* and *skp1-12* mutations supports the idea that Skp1 interacts with multiple proteins to carry out different functions. The *skp1-11* mutation has a greater effect on the degradation of Sic1 than it does on degradation of G_1 cyclins (Bai *et al*., 1996), so this mutation may weaken the interaction of Skp1 with Cdc4 more than it affects the Skp1–Grr1 interaction. The observation that overexpression of *CDC4* suppresses the *skp1-11* mutant (Bai *et al*., 1996) is consistent with this idea. The *skp1-12* mutant, on the other hand, is more defective in degradation of cyclins than Sic1 (Bai *et al*., 1996), and thus may more severely affect the interaction of Skp1 with Grr1 than with Cdc4. As predicted by the model, the *skp1-12* mutation has a more drastic effect on glucose induction of *HXT1* expression than does *skp1-11* (Figure 7 and Table I).

It is not clear which ubiquitin-conjugating enzyme functions with Grr1. Cdc34 is a reasonable candidate because it is required for G_1 cyclin ubiquitination and degradation (Tyers *et al*., 1992; Deshaies *et al*., 1995), and it directly interacts with Cdc53 (Willems *et al*., 1996). However, Cdc34 is not required for glucose induction of *HXT1* expression (Figure 7), suggesting that another ubiquitin-conjugating enzyme may act with Grr1 to ubiquitinate Rgt1 (or its regulator). Cdc34 may not be directly involved in targeting G_1 cyclins for degradation either, because Cln1 and Cln2 are degraded normally in a *cdc34 sic1* mutant (Blondel and Mann, 1996). Cdc34 may be involved only indirectly in degradation of the G_1 cyclins through targeting Sic1 for degradation: degradation of the G_1 cyclins cannot occur until they are phosphorylated by Cdc28, which cannot occur until Sic1 is degraded. Alternatively, Cdc34 may function redundantly with one (or more) of the other 11 yeast ubiquitin-conjugating enzymes in the Grr1–Skp1 complex.

The human homolog of Skp1 (also named Skp1) is in a complex with a protein called Skp2 (Zhang *et al*., 1995). Skp2 may be a counterpart of Grr1 because the two proteins appear to be structurally similar: both contain an F-box and leucine-rich repeats (though the two leucine-rich repeats belong to different families). Also in this protein complex is a cyclin (human cyclin A–CDK2), lending credibility to the suggestion that Skp1–Grr1 of yeast recruits G_1 cyclins.

What is the target of the Grr1–Skp1 complex in the glucose induction pathway?

Grr1 is required for inactivation of the Rgt1 repressor in cells growing on glucose, thus derepressing expression of glucose-induced genes. However, we suspect that Rgt1 is not a direct target of Grr1-mediated proteolysis, because it appears to be present in cells growing both in the presence and absence of glucose: Rgt1 is a repressor in cells growing without glucose, and a transcriptional activator in cells growing on high levels of glucose (Özcan *et al*., 1996). Rather, we imagine that a protein that regulates Rgt1 function is a target of Grr1. One well-known example of this is the regulation of NF-κB, whose inhibitory factor, IF-κB, is degraded by the ubiquitin-mediated proteolysis system (Palombella *et al*., 1994).

It is possible that ubiquitination of Rgt1 (or its regulator) does not target it for protein degradation by the proteasome. Rather, this modification could inhibit the function of Rgt1 without changing its level. There are cases where ubiquitination of a protein alters its function without targeting it to the proteasome for degradation (Hicke and Riezman, 1996; reviewed by Hochstrasser, 1996). However, we favor the idea that Grr1 inhibits Rgt1 function by marking it (or, more likely, its regulator) for degradation, since this is what it appears to do to G_1 cyclins.

Is Grr1p itself regulated by ubiquitin proteolysis?

Grr1 may itself be targeted for degradation by the ubiquitin-conjugating enzyme complex of which it is a part. Grr1 is scarce in yeast cells (Flick and Johnston, 1991; our unpublished results), and we observed a clear correlation between the cellular levels of Grr1 and its ability to interact with Skp1. For example, removing the C-terminus of Grr1 increases its interaction with Skp1 and causes its level to diminish dramatically (Figure 4, line 4 and Figure 5B). Conversely, deleting the F-box or leucine-rich repeats of Grr1 abolishes its ability to interact with Skp1 and causes a concomitant increase in its protein level (Figure 4, lines 2–3). Furthermore, Grr1 isolated from yeast extracts can be ubiquitinated by Cdc34 *in vitro* (A.Banerjee, personal communication). Perhaps ubiquitin-mediated proteolysis contributes to regulation of Grr1 function.

Implications for nutrient sensing and cell cycle regulation

The Grr1–Skp1 interaction is significantly enhanced by high levels of glucose (Figure 5). This may enable yeast cells to couple availability of one of its most important nutrients—glucose—to cell cycle progression as well as to transcriptional events. High levels of glucose are sufficient to trigger a sharp drop in *CLN1* mRNA and protein levels (Tokiwa *et al*., 1994). This can help ensure that yeast, when provided with glucose, resets the critical cell size and grows larger before committing to a new cell cycle (Baroni *et al*., 1994; Tokiwa *et al*., 1994). It seems likely that the Grr1-dependent degradation mechanism contributes to the rapid disappearance of Cln1 upon glucose addition to cells. Glucose also represses *CLN1* and *CLN2* transcription, which contributes to the reduction in Cln protein levels, but this is due to changes in cAMP levels mediated by Ras, and probably does not involve Grr1 (Baroni *et al*., 1994; Tokiwa *et al*., 1994). If the enhancement of the Grr1–Skp1 interaction by glucose has a significant effect on Cln1 degradation, Grr1 could integrate nutrient availability with the cell cycle.

Materials and methods

Strains and plasmids

Yeast strains used are listed in Table II. Plasmids are listed in Table III and their construction is described below.

Plasmids used for Grr1–Skp1 interaction analysis

*pADH–Grr1-*Δ*N*$^{BD\text{-}HA}$ (*pBM2576*). The *GRR1* coding region (amino acids 280–1150) was inserted as a 3.8 kb *Sma*I–*Sal*I fragment from pBM1723 into the *Nco*I (ends blunted with Klenow DNA polymerase) and *Sal*I site of pAS1 vector, which contains sequence encoding hemagglutinin (HA) tag downstream of the Gal4 DNA-binding domain (amino acids 1–147).

*pADH–Grr1-*Δ*NCF*$^{BD\text{-}HA}$ (*pBM3377*). The *GRR1* coding region (amino acids 390–900) was inserted as a 1.5 kb *Bcl*I fragment from pBM2576 into the *Bam*HI site of pAS1.

*pADH–Grr1-*Δ*NC*$^{BD\text{-}HA}$ (*pBM3488*). The plasmid contains fusion between amino acids 280–900 of *GRR1* to Gal4BD of pAS1. It was made by replacing the 2.0 kb *Sac*I fragment of pBM3377 with a 2.2 kb corresponding *Sac*I fragment from pBM2576.

*pADH–Grr1-*Δ*NL*$^{BD\text{-}HA}$ (*pBM2384*). The *GRR1* coding region (amino acids 280–409 and 726–1150) was inserted as a 2.0 kb *Bgl*II fragment from pBM3001 (F.N.Li and M.Johnston, unpublished results) into the *Bam*HI site of pAS1.

*pADH–Grr1-*Δ*N*^{LexA} (*pBM2961*). The *GRR1* coding region (amino acids 158–1150) was inserted as a 3.5 kb *Eco*RI fragment from pBM1679 (Flick and Johnston, 1991) into the *Eco*RI site of pCH435 (Hardy and Pautz, 1996). The resulting plasmid fuses the LexA DNA binding domain (amino acids 1–202) to Grr1, beginning at amino acid 158.

Plasmids used for GRR1–LEU2 overexpression

*pADH–Grr1-*Δ*N*^{AD} (*pBM2868*). The *GRR1* coding region (amino acids 280–1150) was fused to the Gal4 transcription activation domain (776–

Table II. Yeast strains

Strain	Relevant genotype	Source
YM954	*MAT***a** *ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538 trp1-903*	this lab
YM3196	*MATα ade2-101 ura3-52 his3-Δ200 lys2-801 met tyr1 reg1::Leu2 gal80 LEU2::GAL1-lacZ*	this lab
YM4134	*MATα ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538 trp1-903*	this study
YM4575	*MAT***a** *ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538*	this study
YM4576	*MAT***a** *ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538 grr1::hisG*[a]	this study
YM4783	*MATα ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538 grr1::hisG*	this study
YM4887	*MATα ade2-101 ura3-52 his3-Δ200 lys2-801 leu2 gal80-538 gal4-542*	this study
MGG12	*MAT***a** *ade2 ade3 trp1 ura3 his3 cdc53-1*	M.Goebl
MGG15	*MAT***a** *ura3 his3 cdc34-2*	M.Goebl
Y552	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 skp1-11*	S.Elledge
Y554	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 skp1-12*	S.Elledge
Y190	*MAT***a** *ade2-101 his3 ura3-52 leu2-3,-112 trp1-901 gal4 gal80 URA3::Gal-LacZ LYS2::Gal1-His3 cyhR*	S.Elledge
L40	*MAT***a** *ade2-101 his3-200 trp1-901 leu2-3,-112 gal4 gal80 Lys2::(LexO)$_4$-Gal1-His3 Ura3::(LexO)$_8$-GAL1-lacZ*	S.Hollenberg

[a]The *grr1* disruption, which removes amino acids 24–1151 (inclusive), is present in plasmid pBM2101 (Flick and Johnston, 1991).

881) by inserting a 3.8 kb *Nco*I–*Sal*I fragment from pBM2576 into the same sites of pACTII vector (Durfee *et al*., 1993).

pADH–Grr1-ΔNC^{AD} (*pBM3003*). The plasmid contains a fusion of the *GRR1* coding region (amino acids 280–920) to the Gal4 transcription activation domain (776–881) and was made by two steps. First, pBM1775 (Grr1-ΔNC916) was gap-repaired with a fragment containing the upstream and N-terminus regions of *GRR1* from pBM2077 to generate pBM2901 (Grr1-ΔC916). Second, a 1.9 kb *Bgl*II–*Sal*I fragment encoding amino acids 280–916 of Grr1 from pBM2901 was inserted into the *Bam*HI–*Xho*I site of pACT to generate pBM3003.

Plasmids used for Grr1 in vitro translation

pT7–Grr1-ΔN (*pBM3383*). The *GRR1* coding region (amino acids 280–1150) was inserted as a 3.8 kb *Nco*I–*Xba*I fragment from pBM2868 between the same sites of pcDNA3.1/Zeo (+) (Invitrogen).

pT7–Grr1-ΔNC; *pT7–Grr1-ΔNCF* (*pBM3429; pBM3489*). A 400 bp *Nco*I fragment containing the T7 promoter from pBM3383 was inserted into the *Nco*I site of pBM3488 and pBM3377 respectively.

Plasmids for in vitro binding assays

GST–Skp1 (*pBM3391*). A 0.7 kb *Bgl*II fragment from the Skp1 two-hybrid clone (*pBM3280*) was inserted into the *Bam*HI site of pGEX-3X (Pharmacia). The resulting plasmid fuses GST protein to Skp1 beginning at amino acid 13.

SKP1–pRS426 (*pBM3490*). A 2.7 kb *Bam*HI fragment from pCB6 (Bai *et al*., 1996) (generous gift of S.Elledge) containing the genomic region of *SKP1* was inserted into the *Bam*HI site of pRS426 (Christianson *et al*., 1992)

Antibodies

Monoclonal antibody against the Gal4 transcription activation domain, 7E10-G10, was a generous gift from G.Sprague (Printen and Sprague, 1994). Monoclonal antibody against hemagglutinin (HA) epitope tag, 12CA5, was obtained from BAbCo.

Two-hybrid screen

The Grr1 bait plasmid BD-Grr1 (pBM2576, *TRP1* marker) and the yeast two-hybrid cDNA library (in pACT, *LEU2* marker, gift of S.Elledge) were co-transformed into reporter strain Y190 (Harper *et al*., 1993). Leu$^+$ Trp$^+$ colonies that turned blue in the β-galactosidase filter assay could contain cDNA clones encoding a Grr1-interacting protein. Their cDNA clones were recovered and reintroduced into yeast along with either the same Grr1 bait plasmid or the plasmid encoding control bait (BD-Snf1). Only those clones that interacted specifically with Grr1 bait protein were studied further. The cDNA insert was sequenced from both ends using oligonucleotides OM520 (5′-AACTATCTATTCGATG-3′) and OM521 (5′-CACAGTTGAAGTGAAC-3′). For the two-hybrid screen using LexA–Grr1 (pBM2961) as bait, three modifications of the procedures were made. Yeast strain L40 was used instead of Y190; transformants were selected on medium lacking leucine, tryptophan and histidine; LexA–Rfc3 (generous gift from X.Li and P.Burger) was used as the alternative control bait.

Table III. Plasmids

Plasmid	Relevant characteristics	Source
pAS1	*pADH–Gal4BD–HA-Trp1-2*μ	S.Elledge
pACT	*pADH–Gal4AD–LEU2-2*μ	S.Elledge
pACTII	*pADH–Gal4AD–LEU2-2*μ	S.Elledge
BM1679	*GRR1–URA3–CEN* (in Ycp50)	this lab
BM1723	*GRR1-ΔN–URA3–CEN* (in pUN70)	this lab
BM2576	*pADH–GRR1-ΔN$^{BD\text{-}HA}$* (in pAS1)	this study
BM3377	*pADH–GRR1-ΔNCF$^{BD\text{-}HA}$* (in pAS1)	this study
BM3488	*pADH–GRR1-ΔNC$^{BD\text{-}HA}$* (in pAS1)	this study
BM2384	*pADH–GRR1-ΔNL$^{BD\text{-}HA}$* (in pAS1)	this study
BM2961	*pADH–GRR1-ΔN^{LexA}-TRP1-2*μ	this study
BM2868	*pADH–GRR1-ΔN^{AD}* (in pACTII)	this study
BM3003	*pADH–GRR1-ΔNC^{AD}* (in pACT)	this study
BM3383	*pT7–GRR1-ΔN*	this study
BM3429	*pT7–GRR1-ΔNC*	this study
BM3489	*pT7–GRR1-ΔNCF*	this study
pCB6	*SKP1–LEU2* (in pRS415)	S.Elledge
BM3490	*SKP1–URA3* (in pRS426)	this study
BM3280	*pADH–Skp1-N13^{AD}* (in pACT)	this study
BM3391	*GST–SKP1* (in pGEX-3X)	this study

β-Galactosidase assays

β-Galactosidase activities were measured in permeabilized mid-log phase cells as previously described (Özcan and Johnston, 1995) and are the averages of four to six assays of three independent transformants. The two-hybrid strains were grown on selective YNB-2% glucose or raffinose liquid media before the assays. For determination of *HXT1::lacZ* induction in *skp1^{ts}* strains, three independent cultures were pregrown at 30°C to mid-log phase on YNB-2% galactose without uracil, then shifted to 37°C for 60 min. Glucose was then added to the media (4%, w/v) and samples of the culture (kept at 37°C) were assayed at various times for β-galactosidase activity.

Expression of GST fusion proteins

E.coli strain DH5α was used as the host for GST fusion plasmids. The expression of fusion proteins was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside, 1 mM final concentration). Bacterial pellets were resuspended in ice-cold lysis buffer (1% Triton X-100, 1 mM PMSF, 2 μM pepstatin, 0.6 μM leupeptin in PBS buffer) and sonicated on ice three times for 30 s using a micro-tip. Extracts were cleared by centrifugation at 10 000 r.p.m. for 20 min in a Sorvall SS-34 rotor. Cleared extracts were then incubated with glutathione–Sepharose beads (Sigma) at 4°C overnight. The protein-bound beads were washed with, and resuspended in, PBS buffer. For subsequent binding assays, the concentration of GST fusion proteins in the slurry of glutathione–agarose beads was determined by SDS–PAGE and Coomassie blue staining.

In vitro binding assay

^{35}S-labeled Grr1 protein was made by *in vitro* transcription and translation in a 50 μl reaction (Promega TNT, according to the supplier's protocol).

Ten µl of the reaction was mixed with an equal amount of GST–Skp1 or GST–Sepharose beads in a final volume of 100 µl binding buffer (10 mM Tris–HCl, pH 7.5. 250 mM NaCl, 5 mM EDTA, 1% Triton X-100). The reaction was incubated for 2 h at 4°C with gentle agitation. After three washes with 1 ml of binding buffer (in the last wash, NaCl concentration was changed to 500 mM in the buffer), beads were boiled in SDS loading dye and resolved by SDS–PAGE. Bound proteins were detected by fluorography.

Protein extracts

Protein extracts were made as follows: yeast cultures were harvested at OD_{590} of ~1.0 and washed once in cold Tris–HCl buffer, pH 7.5. The cells were then resuspended in Yeast Lysis Buffer (10% glycerol, 1% NP-40 in TBS buffer, pH 7.5, supplemented with protease inhibitors: 1 mM PMSF, 2 µM pepstatin and 0.6 µM leupeptin) and lysed by vortexing with an equal volume of glass beads for 5 min in the cold room. The lysates were cleared by centrifugation at 10 000 r.p.m. for 20 min in a Sorvall SS-34 rotor. The protein concentrations of the lysate were determined by the Bradford assay (Bio-Rad). The average protein concentration of lysates prepared in this way is 1.0–2.0 µg/µl.

Immunoprecipitation and Western blots

Immunoprecipitations were carried out by incubating 200–400 µl of yeast extract (containing 0.5–1.0 mg of total yeast protein) with primary antibody for 4 h at 4°C with gentle rocking. Typical reactions used 50 µl antibody serum with additional Yeast Lysis Buffer to adjust the final volume to 500 µl. Fifty µl of pre-washed protein A–Sepharose beads (Sigma) were then added and incubated for another 4 h at 4°C. The beads were then washed three times with 1 ml of Yeast Lysis Buffer, resuspended in SDS loading dye, and resolved by SDS–PAGE. For Western blots, proteins were transferred to PVDF membrane (Millipore) by a Mini Protean Transfer Apparatus (Bio-Rad) at 300 mA overnight. The proteins were detected using an enhanced chemiluminescence system (ECL, Amersham). Dilution of the antibodies for Western blots were 1:100 for anti-AD antibody and 1:500 for anti-HA antibody.

Imaging processing

All images presented here were scanned with an Epson ES-1200C Scanner and processed using Photoshop 3.05 software on a Macintosh platform.

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References

Bai,C., Sen,P., Hofmann,K., Ma,L., Goebl,M., Harper,J.W. and Elledge,S.J. (1996) *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, **86**, 263–274.

Bailey,R.B. and Woodword,A. (1984) Isolation and characterization of a pleiotropic glucose repression resistant mutant of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **193**, 507–512.

Baroni,M.D., Monti,P. and Alberghina,L. (1994) Repression of growth-regulated G1 cyclin expression by cyclic AMP in budding yeast. *Nature*, **371**, 339–342.

Barral,Y., Jentsch,S. and Mann,C. (1995) G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. *Genes Dev.*, **9**, 399–409.

Blondel,M. and Mann,C. (1996) G2 cyclins are required for the degradation of G1 cyclins in yeast. *Nature*, **384**, 279–282.

Christianson,T.W., Sikorski,R.S., Dante,M., Shero,J.H and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.

Conklin,D.S., Kung,C. and Culbertson,M.R. (1993) The *COT2* gene is required for glucose-dependent divalent cation transport in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 2041–2049.

Connelly,C. and Hieter,P. (1996) Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell*, **86**, 275–285.

Deshaies,R.J., Chau,V. and Kirschner,M. (1995) Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J.*, **14**, 303–312.

Durfee,T., Becherer,K., Chen,P.L., Yeh,S.H., Yang,Y.Z., Kilburn,A.E., Lee,W.H. and Elledge,S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555–569.

Erickson,J.R. and Johnston,M. (1994) Suppressors reveal two classes of glucose repression genes in the yeast *Saccharomyces cerevisiae*. *Genetics*, **136**, 1271–1278.

Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Flick,J.S. and Johnston,M. (1991) *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. *Mol. Cell. Biol.*, **11**, 5101–5112.

Goebl,M.G., Yochem,J., Jentsch,S., McGrath,J.P., Varshavsky,A. and Byers,B. (1988) The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science*, **241**, 1331–1335.

Hardy,C.F. and Pautz,A. (1996) A novel role for Cdc5p in DNA replication. *Mol. Cell. Biol.*, **16**, 6775–6782.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Hicke,L. and Riezman,H. (1996) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell*, **84**, 277–287.

Hochstrasser,M. (1995) Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.*, **7**, 215–223.

Hochstrasser,M. (1996) Protein degradation or regulation: Ub the judge. *Cell*, **84**, 813–815.

Jackson,P.K. (1996) Cell cycle: cull and destroy. *Curr. Biol.*, **6**, 1209–1212.

Johnston,M. and Carlson,M. (1993) Regulation of carbon and phosphate utilization. In Broach,J., Jones,E.W. and Pringle,J. (eds), *The Biology of the Yeast Saccharomyces*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 193–281.

Kim,Y.J., Francisco,L., Chen,G.C., Marcotte,M. and Chan,C.S. (1994) Control of cellular morphogenesis by the Ip12/Bem2 GTPase-activating protein: possible role of protein phosphorylation. *J. Cell Biol.*, **127**, 1381–1394.

King,R.W., Deshaies,R.J., Peters,J.-M. and Kirschner,M.W. (1996) How proteolysis drives the cell cycle. *Science*, **274**, 1652–1659.

Kobe,B. and Deisenhofer,J. (1994) The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.*, **19**, 415–421.

Lanker,S., Valdivieso,M.H. and Wittenberg,C. (1996) Rapid degradation of the G_1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science*, **271**, 1597–1601.

Mathias,N., Johnson,S.L., Winey,M., Adams,A.E.M., Goetsch,L., Pringle,J.R., Byers,B. and Goebl,M. (1996) Cdc53 acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins. *Mol. Cell. Biol.*, **16**, 6634–6643.

Neer,E.J., Schmidt,C.J., Nambudripad,R. and Smith,T.F. (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature*, **371**, 297–300.

Özcan,S. and Johnston,M. (1995) Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose. *Mol. Cell. Biol.*, **15**, 1564–1572.

Özcan,S., Leong,T. and Johnston,M. (1996) Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol. Cell. Biol.*, **16**, 6419–6426.

Palombella,V.J., Rando,O.J., Goldberg,A.L. and Maniatis,T. (1994) The ubiquitin–proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell*, **78**, 773–785.

Printen,J.A. and Sprague,G.F.,Jr. (1994) Protein–protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade. *Genetics*, **138**, 609–619.

Ronne,H. (1995) Glucose repression in fungi. *Trends Genet.*, **11**, 12–17.

Salama,S.R., Hendricks,K.B. and Thorner,J. (1994) G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover. *Mol. Cell. Biol.*, **14**, 7953–7966.

Schwob,E., Böhm,T., Mendenhall,M.D. and Nasmyth,K. (1994) The B-type cyclin kinase inhibitor $p40^{sic1}$ controls the G1 to S transition in *S.cerevisiae*. *Cell*, **79**, 233–244.

Tokiwa,G., Tyers,M., Volpe,T. and Futcher,B. (1994) Inhibition of G1 cyclin activity by the Ras/cAMP pathway in yeast. *Nature*, **371**, 342–345.

Trumbly,R.J. (1992) Glucose repression in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **6**, 15–21.

Tyers,M., Tokiwa,G., Nash,R. and Futcher,B. (1992) The Cln3–Cdc28 kinase complex of *S.cerevisiae* is regulated by proteolysis and phosphorylation. *EMBO J.*, **11**, 1773–1784.

Vallier,L.G., Coons,D., Bisson,L.F. and Carlson,M. (1994) Altered regulatory responses to glucose are associated with a glucose transport defect in *grr1* mutants of *Saccharomyces cerevisiae*. *Genetics*, **136**, 1279–1285.

Willems,A.R., Lanker,S., Patton,E.E., Craig,K.L., Nason,T.F., Mathias,N., Kobayashi,R., Wittenberg,C. and Tyers,M. (1996) Cdc53 targets phosphorylated G_1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell*, **86**, 453–463.

Wittenberg,C.K., Sugimoto,K. and Reed,S.I. (1990) G1-specific cyclins of *S.cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{CDC28}$ protein kinase. *Cell*, **62**, 225–237.

Yaglom,J., Linskens,M.H.K., Sadis,S., Rubin,D.M., Futcher,B. and Finley,D. (1995) $p34^{CDC28}$-mediated control of Cln3 cyclin degradation. *Mol. Cell. Biol.*, **15**, 731–741.

Yochem,J. and Byers,B. (1987) Structural comparison of the yeast cell division cycle gene *CDC4* and a related pseudogene. *J. Mol. Biol.*, **195**, 233–245.

Zhang,H., Kobayashi,R., Galaktionov,K. and Beach,D. (1995) $p19^{Skp1}$ and $p45^{Skp2}$ are essential elements of the cyclin A–CDK2 S-phase kinase. *Cell*, **82**, 915–925.

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