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A Novel Degron-mediated Degradation of the RTG Pathway Regulator, Mks1p, by SCF^{Grr1} [D]

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Yeast cells respond to mitochondrial dysfunction by altering the expression of a subset of nuclear genes, a process known as retrograde signaling (RS). RS terminates with two transcription factors, Rtg1p and Rtg3p. One positive regulator, Rtg2p, and four negative regulators, Lst8p, Mks1p, and the redundant 14-3-3 proteins, Bmh1p and Bmh2p, control RS upstream of Rtg1/3p. Mks1p is negatively regulated by binding to Rtg2p and positively regulated when bound to Bmh1/2p. Here we report that Grr1p, a component of the SCF^{Grr1} E3 ubiquitin ligase, modulates RS by affecting Mks1p levels. Grr1p polyubiquitinates Mks1p not bound to either Rtg2p or to Bmh1/2p, targeting it for degradation. An acidic domain region of Mks1p constitutes the portable Mks1p degron sequence. We have isolated dominant mutations in Grr1p leading to increased Mks1p degradation. These mutations result in a gain of positive charge on the concave surface of the leucine rich repeat (LRR) domain of Grr1p, the proposed substrate binding site. We propose that Mks1p is a central player of RS and is acted upon by multiple regulators of the pathway.

INTRODUCTION

The retrograde response is a mitochondria-to-nucleus signaling pathway that monitors and transmits changes in mitochondrial function to specific changes in nuclear gene expression (Butow and Avadhani, 2004). This response is, for the most part, adaptive, affecting diverse cellular activities that include metabolic, nutrient sensing, transport and stress pathways (Epstein *et al.,* 2001; Biswas *et al.,* 2003). These activities are adjusted to accommodate cells to the alterations in the mitochondrial state, for instance, to the loss of respiratory activity. In animal cells, mitochondrial dysfunctions often initiate changes in intracellular Ca^{2+} dynamics that lead to changes in nuclear gene expression via the activation of transcription factors, e.g., NFκB and NFAT (Luo *et al.,* 1997; Biswas *et al.,* 1999, 2003). In the budding yeast, *Saccharomyces cerevisiae*, the loss of respiratory activity results in increased expression of a defined set of nuclear genes encoding proteins that participate in activities that include anaplerotic pathways, peroxisome biogenesis, small molecule transport systems and pleiotropic drug resistance (Hallstrom and Moye-Rowley, 2000; Epstein *et al.,* 2001).

Expression of some retrograde responsive genes in yeast, such as *CIT2*, encoding a glyoxylate cycle isoform of citrate synthase, requires three regulatory factors, Rtg1p, Rtg2p, and Rtg3p (Liao and Butow, 1993; Jia *et al.,* 1997). This requirement places these genes in the RTG pathway, in contrast to other retrograde responsive genes whose elevated expression in cells with dysfunctional mitochondria is essentially independent of the *RTG* genes (Epstein *et al.,* 2001). Rtg1p and Rtg3p are bHLH/Zip transcription factors that bind to the promoter region of RTG target genes (Jia *et al.,* 1997). When the RTG pathway is activated, a hyperphosphorylated form of Rtg3p sequestered in the cytoplasm with Rtg1p becomes partially dephosphorylated, and both transcription factors translocate to the nucleus (Sekito *et al.,* 2000). These processes require Rtg2p, a novel cytoplasmic protein with an N-terminal ATP binding domain whose integrity is required for Rtg2p function (Liao and Butow, 1993; Liu *et al.,* 2003). The RTG pathway can also be activated by inhibition of TOR (target of rapamycin) kinase activity (Komeili *et al.,* 2000). The intersection of these pathways underscores the intimate relation between mitochondrial function and nutrient sensing, one of the important activities of TOR signaling.

A key feature of the RTG pathway is its role in reconfiguring metabolism to meet the special demands of respiratory deficient cells. For instance, transcriptional regulation of the Krebs cycle genes, *CIT1*, *ACO1*, *IDH1,* and *IDH2* switches from control by the HAP transcriptional complex to the Rtg1/3p complex in respiratory-deficient cells (Liu and Butow, 1999). The products of these genes produce α-ketoglutarate, the direct precursor of glutamate. Consequently, mutations in any one of the *RTG* genes lead to glutamate auxotrophy in cells that are respiratory deficient (Liu and Butow, 1999). Glutamate is also a potent repressor of the RTG pathway (Liu and Butow, 1999; Sekito *et al.,* 2002), underscoring the importance of the pathway in glutamate homeostasis. These findings highlight how the RTG pathway functions to regulate the changing metabolic needs of cells with altered mitochondrial function.

Additional regulatory factors have been identified that act between Rtg2p and Rtg1/3p. These include Mks1p, Lst8p, and the 14-3-3 proteins Bmh1p and Bmh2p, all of which negatively regulate the RTG pathway (Liu *et al.,* 2001; Dilova *et al.,* 2002; Sekito *et al.,* 2002; Tate *et al.,* 2002; Liu *et al.,* 2003). In cells that either lack or have mutant forms of these proteins, *RTG*-dependent gene expression is constitutive and is

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Abbreviations used: RS, retrograde signaling.

not dependent on Rtg2p. Recent studies have provided additional insights into the regulation of the RTG pathway involving a dynamic interplay among Mks1p, Rtg2p, and Bmh1/2p (Liu *et al.*, 2003). Although the specific mechanism of how Mks1p, a phosphoprotein, acts as a negative regulator has not yet been established, what is clear is that when the RTG pathway is activated, Mks1p is largely dephosphorylated and is sequestered by Rtg2p, effectively relieving the negative regulation of the RTG pathway. Conversely, when the RTG pathway is repressed, for example, in respiratory-competent cells, or by the addition of glutamate to the medium, Mks1p is hyperphosphorylated and is in a complex with Bmh1/2p. Mks1p in that complex has been suggested to be the active, negative regulator of the RTG pathway (Liu *et al.*, 2003).

The main objective of this study was identification and characterization of a new regulatory factor of the RTG pathway. Here we show that Mks1p levels are regulated by Grr1p, the F-box component of the SCF^{Grr1} (Skp1-Cullin-F box) E3 ubiquitin ligase (Deshaies, 1999), and that modulation of Mks1p levels can affect the regulation of the RTG pathway. We have isolated dominant *GRR1* mutants with single amino acid changes within the putative substrate binding site localized to the concave surface of the Grr1p leucine-rich repeat (LRR) domain. These changes result in a net increase in positive charge and lead to instability of Mks1p and activation of the RTG pathway. We found that the Bmh1/2p act as negative regulators of the RTG pathway by protecting Mks1p from degradation by Grr1p. We have identified a novel, portable Mks1p degron sequence, which is an acidic domain in the central region of the protein. Finally, we provide evidence that the form of Mks1p targeted for degradation by Grr1p is not bound to either Rtg2p or to Bmh1/2p.

MATERIALS AND METHODS

Strains and Plasmids

Strains and plasmids used in this study are available at *Molecular Biology of the Cell* Online.

Growth Media

Yeast strains were grown at 30°C in YPD medium (1% yeast extract, 2% bactopeptone, and 2% dextrose), YNBcasD (0.67% yeast nitrogen base, 1% casamino acids, and 2% dextrose), SCRaffGal (0.67% yeast nitrogen base, 0.065% CSM-uracil-leucine dropout mix [Bio101, Carlsbad, CA], 2% raffinose, and 2% galactose), or minimal YNBD medium (0.67% yeast nitrogen base, 2% dextrose) with or without glutamate (concentrations indicated in the text and figures). MG132 (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide and applied at a final concentration of 50 μM.

EMS Mutagenesis and Cloning of GRR1 Dominant Mutants

EMS mutagenesis was conducted as described in Liu *et al.* (2001). Cloning of dominant *rtg2*Δ bypass mutations was described in Liu *et al.* (2003). Briefly, a yeast genomic DNA library was constructed from strain G15 and transformed into *rtg2*Δ cells (PSY142-rtg2) to recover the mutant gene conferring the *rtg2*Δ bypass phenotype. This new *rtg2*Δ bypass mutant was due to a mutation in *GRR1*. *GRR1* was then cloned by PCR from a collection of 18 other dominant *rtg2* bypass mutants to determine whether these mutants harbor *GRR1* mutations. Altogether, eight *GRR1* mutations were uncovered, with five due to change of glutamic acid at position 522 to lysine (E522K), one E548K mutation, one D657N mutation, and one S444R mutation.

Total mRNA Isolation and Northern Blot Analysis

Total yeast RNA was isolated from 50-ml logarithmic-phase cultures, fractionated on 1·2% agarose gels, transferred to Nylon membrane and hybridized at 65°C with probes specific for transcripts of the *CIT2* and *ACT1* genes. Hybridization signals were detected with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

β-galactosidase Activity Assays for CIT2-lacZ Reporter Gene Expression

Liquid cultures were inoculated with a pool of several independent transformants in YNBcasD medium and grown to OD_{600} 0.6–0.8. The preparation of cell extracts and β-galactosidase assays were carried out as described by Rose *et al.* (1990).

Cell Extracts, Immunoblotting, Immunoprecipitations, and Ubiquitin Assay

Whole cell extracts were prepared and coimmunoprecipitation experiments were performed as described by Sekito *et al.* (2000) except for the following changes: for ubiquitination assay of Mks1p, buffer A (20 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 0.7% deoxycholate, 0.1% SDS, and protease inhibitors) was used; for interaction between Mks1p and Bmh1p, Rtg2p, or Grr1p, buffer B was used (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors). Samples were resolved by SDS-PAGE. Detection of HA- or myc-tagged proteins on Western blots was conducted as described (Liu *et al.*, 2003). Anti-ubiquitin antibody (P4D1, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect ubiquitinated species of Mks1p.

Cycloheximide Chase Assay

Cells expressing indicated HA-tagged proteins were grown in liquid YNBcasD medium to OD_{600} ~1. Cycloheximide (50 μg/ml) was added to start the chase. Every 15 or 20 min, a 1-ml aliquot of the cell culture was withdrawn, and the cell pellets were subject to TCA precipitation as described (Sekito *et al.*, 2000).

In Vitro Translation of Mks1p and GST Pulldown Assay with GST or GST-Grr1p

MKS1 and *MKS1 (ΔAD)* cloned in pET24a (Novagen, Madison, WI) were in vitro translated and labeled with ^{35}S-methionine using the TnT T7 Quick coupled transcription/translation labeling kit (Promega, Madison, WI). GST and GST-Grr1p was expressed in bacteria BL21(DE3) cells (Novagen) and purified using Sepharose 4B beads (Amersham Pharmacia, Piscataway, NJ). For GST pulldown assay, GST fusion proteins bound to beads were equilibrated with GST buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 0.05% Nonidet NP-40, 5 mM dithiothreitol, 10% glycerol, 50 μg/ml bovine serum albumin), and the binding reactions were performed in the same buffer. After incubation of GST or GST-Grr1p with ^{35}S-labeled Mks1p or Mks1p (ΔAD) for 2 h at 4°C, the beads were washed five times with 1 ml GST buffer. Bound proteins were eluted by boiling in protein loading buffer, separated by SDS-PAGE, and dried gels were analyzed by autoradiography.

RESULTS

Dominant GRR1 Mutants Bypass the rtg2Δ Mutation

To search for new regulatory components of the RTG pathway, we used a genetic screen for *rtg2*Δ bypass mutants that was successfully used to identify the RTG regulators, Lst8p, Mks1p, and Bmh1/2p (Liu *et al.*, 2001; Sekito *et al.*, 2002; Liu *et al.*, 2003). The current screen revealed eight dominant *GRR1* mutants representing four different alleles, *GRR1-(1-4)*. *GRR1* encodes a subunit of the SCF^{Grr1} E3 ubiquitin ligase complex. As is shown for two of the alleles, *GRR1-1* and *GRR1-2*, these mutants confer an *rtg2*Δ bypass phenotype to the expression of a *CIT2-lacZ* reporter gene (Figure 1A). Expression of these mutant alleles from centromeric plasmids restored the block in *CIT2* reporter gene expression in *rtg2*Δ *GRR1* cells, indicating that they are dominant mutations (Figure 1B). Neither mutant was able to rescue *CIT2* reporter gene expression in *rtg2*Δ *rtg3*Δ cells, showing that they affect the RTG pathway downstream of *RTG2* but upstream of *RTG1/3*. Similar results were obtained for the *GRR1-3* and *GRR1-4* mutants (unpublished data). Finally, none of the mutants affect the steady state level of Grr1p, and overexpression of wild-type *GRR1* from a 2 μm plasmid failed to bypass the *rtg2*Δ mutation (unpublished data).

Mks1p Is Unstable in rtg2Δ GRR1-1 and rtg2Δ GRR1-2 Mutant Cells

Sequence analysis of the *GRR1* mutant alleles revealed that each contains a single amino acid change in the LRR domain of Grr1p, resulting in a net gain of one or two positive

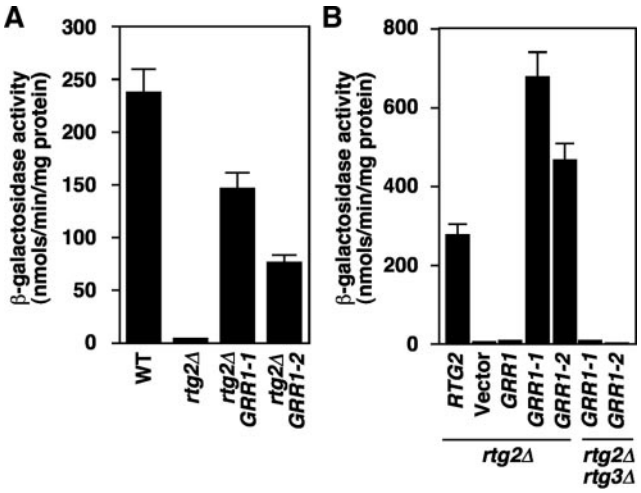

Figure 1. GRR1 mutations activate Rtg2p-independent *CIT2-lacZ* reporter gene expression. (A) Two dominant *GRR1* mutations, *GRR1-1* and *GRR1-2*, restore *CIT2-lacZ* reporter gene expression in *rtg2*Δ cells. Wild-type (WT) and mutant haploid strains as indicated were examined for *CIT2-lacZ* expression as described in *Materials and Methods*. (B) The *GRR1-1* and *GRR1-2* mutant alleles are dominant and their *rtg2*Δ bypass phenotype is dependent on *RTG3*. *rtg2*Δ and *rtg2*Δ *rtg3*Δ mutant cells transformed with centromeric plasmids containing *RTG2*, *GRR1*, *GRR1-1*, *GRR1-2* or no insert (vector) were examined for *CIT2-lacZ* expression.

charges (E522K in *GRR1-1*, E548K in *GRR1-2*, S444R in *GRR1-3*, and D657N in *GRR1-4*; Figure 2A). LRR domains are generally involved in protein-protein interactions. The structure of the LRR domain from porcine liver ribonuclease inhibitor reveals a horseshoe-shaped solenoid with a concave surface of parallel β-sheets and a convex surface of α-helices (Kobe and Deisenhofer, 1995). The mutations in the Grr1p LRR domain map to the predicted concave surface of the protein (Figure 2B), which has been proposed to be the binding site for Cln2p (Hsiung *et al.*, 2001).

Because substrates for E3 ubiquitin ligases are generally phosphoproteins (Deshaies, 1999), a candidate substrate for Grr1p is the RTG negative regulator, Mks1p. Mks1p is hyperphosphorylated when the retrograde pathway is shut down, for example, in *rtg2*Δ cells (Sekito *et al.*, 2002). We therefore asked whether the *GRR1* dominant mutants might bypass the *rtg2*Δ mutation because of increased degradation of Mks1p. Accordingly, we examined the levels of a functional, C-terminal HA-tagged derivative of Mks1p expressed from a centromeric plasmid under the control of the *ADH1* promoter in *rtg2*Δ cells at various times after the addition of cycloheximide. In otherwise wild-type *rtg2*Δ cells, Mks1p-HA was relatively stable over the 60-min cycloheximide chase period (Figure 2C and Supplementary Figure S1). In contrast, in both *rtg2*Δ *GRR1-1* and *rtg2*Δ *GRR1-2* mutant cells, Mks1p-HA was unstable, as most of it disappeared by 30 min after cycloheximide addition. These findings are consistent with the notion that the *rtg2*Δ bypass phenotype in *GRR1-1/2* mutant cells is due to Mks1p degradation. Increased degradation of Mks1p-HA in *rtg2*Δ *GRR1-1* cells was also observed in a Gal shut-off assay in which Mks1p-HA expression was placed under the control of the galactose-inducible *GAL1* promoter (Supplementary Figure S2). This indicates that the Mks1p-HA instability observed in Figure 2C is not an artifact due to the growth inhibitory effects of cycloheximide.

The Grr1p-dependent degradation of Cln2 has been well studied (Lanker *et al.*, 1996; Hsiung *et al.*, 2001). To determine whether the *GRR1* dominant mutants also affect Cln2 stability, we carried out a Gal shut-off assay in which Cln2-HA expression was placed under the control of the *GAL1* promoter. Neither the *GRR1-1* or *GRR1-2* mutation has an affect on Cln2-HA stability (Supplementary Figure S3), indicating the specificity of the *GRR1-1/2* mutations toward Mks1p.

In wild-type cells with robust RTG pathway activity, Mks1p is hypophosphorylated and in a complex with Rtg2p (Liu *et al.*, 2003). To determine the stability of that form of Mks1p in *GRR1-1* and *GRR1-2* cells, Mks1p-HA was expressed as above, and in the same cells, Rtg2p was overexpressed from a wild-type *RTG2* allele integrated at the *LEU2* locus under the control of the strong *TEF1* promoter so as to drive most, if not all, of the Mks1p-HA into a complex with Rtg2p. The validity of this strategy is indicated by the fact that the glutamate auxotrophy resulting from Mks1p-HA overexpression (due to down-regulation of the RTG pathway) can be completely reversed by overexpression of Rtg2p (unpublished data). As shown in Figure 2D, Mks1p-HA expressed in *GRR1-1* and *GRR1-2* cells is now stable in transformants overexpressing Rtg2p. As shown previously (Sekito *et al.*, 2002; Liu *et al.*, 2003), Mks1p is hypophosphorylated in cells coexpressing Rtg2p. The protection effect of overexpression of Rtg2p on Mks1p stability is unlikely to be indirect, for example, by inhibiting SCF^{Grr1}, because overexpression does not affect Cln2 stability (Supplementary Figure S3).

To show directly that the *rtg2*Δ bypass phenotype of *GRR1-1/2* mutants is specifically the result of increased degradation of Mks1p, we increased the expression of Mks1p in *GRR1-1* cells, reasoning that the *rtg2*Δ bypass phenotype might be suppressed by saturation of the Mks1p degradation system. *GRR1-1* cells were transformed with a centromeric plasmid expressing Mks1p from its own promoter (CEN *MKS1*), from the stronger *ADH1* promoter (*ADH1p-MKS1*), or from a multicopy 2μ plasmid also under the control of the *ADH1* promoter (2μ *ADH1p-MKS1*). Down-regulation of the RTG pathway in those cells was then scored by the appearance of glutamate auxotrophy. In wild-type cells, expression of Mks1p from *ADH1p-MKS1* or 2μ *ADH1p-MKS1* is sufficient to repress the RTG pathway (Figure 2E). In *rtg2*Δ cells, which show a slightly leaky glutamate auxotrophy, full repression of the RTG pathway can be achieved when Mks1p was expressed from CEN *MKS1*. In contrast, significant suppression of the *rtg2*Δ bypass phenotype in *GRR1-1* mutant cells requires that Mks1p be expressed from 2μ *ADH1p-MKS1*. These results show that there is an Mks1p dosage-dependent reversal of the *GRR1-1* mutant phenotype, suggesting that the *rtg2*Δ bypass phenotype of the *GRR1-1/2* dominant mutants is specifically because of an increase in the degradation of Mks1p.

The level of Mks1p expressed from the *ADH1* promoter is about fivefold higher than that expressed from its own promoter (unpublished data). To exclude the possibility that instability of Mks1p in Figure 2C is due to Mks1p overexpression, we examined the stability of Mks1p expressed from its own promoter and found it is unstable in *rtg2*Δ *GRR1-1* cells (Figure 2F and Supplementary Figure S4). Moreover, the steady state level of Mks1p expressed from its own promoter was reduced by >10-fold in *rtg2*Δ *GRR1-1* cells (Supplementary Figure S5), consistent with the notion that *rtg2*Δ bypass phenotype of the *GRR1-1* mutation is due to accelerated Mks1p degradation.

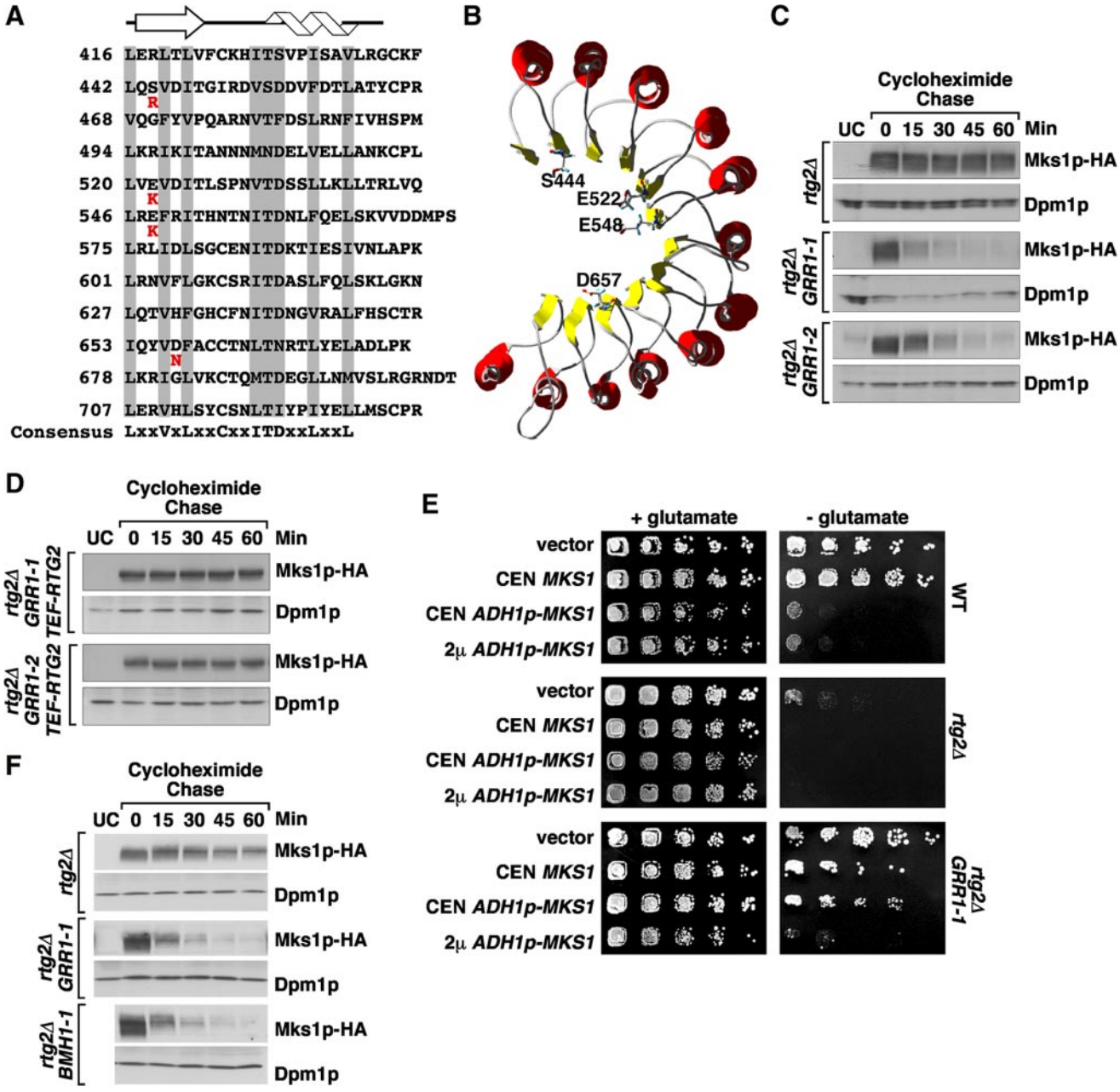

Figure 2. *GRR1* dominant mutations destabilize Mks1p in *rtg2*Δ cells. (A) Sequence alignment of the Grr1p leucine-rich repeats (LRR). The conserved residues are highlighted in gray and the consensus sequence shown at the bottom indicates amino acid residues present in at least five of the repeats. The altered residue of each of the four dominant mutations of *GRR1-(1–4)* is indicated in red underneath the wild-type residue. The predicted secondary structure (helix for α-helix and arrow for β-sheet) is indicated at the top. (B) The dominant *GRR1* mutations localize to the concave surface of the LRR of Grr1p. The modeled 3D structure is adapted from Hsiung *et al.* (2001). The residues mutated in the dominant *GRR1* mutants are indicated by showing their side chains. (C) Mks1p expressed from the *ADH1* promoter is unstable in *rtg2*Δ *GRR1-1* and *rtg2*Δ *GRR1-2* cells. Mks1p stability was examined in indicated cells using cycloheximide chase assay as described in *Materials and Methods*.. Mks1p-HA levels were determined by probing Western blots with anti-HA antibody. Dpm1p, dolichol phosphate mannose synthase, was used as loading control. UC, untagged control. (D) Rtg2p protects Mks1p from degradation in *GRR1-1* and *GRR1-2* mutants. Mks1p stability was examined following a cycloheximide chase in *rtg2*Δ *GRR1-1* and *rtg2*Δ *GRR1-2* cells, each containing a copy of *RTG2* integrated at the *LEU2* locus under the control of the *TEF1* promoter. (E) Overexpression of *MKS1* reverses the *rtg2*Δ bypass phenotype of the *GRR1-1* mutation. Serial dilutions of culture of indicated cells were prepared and cells were spotted onto solid YNBD medium with or without 0.02% glutamate. Cells were grown for three days at 30°C. (F) Stability assay of Mks1p-HA expressed from its own promoter. Mks1p stability was examined using cycloheximide chase assay.

Bmh1/2p protect Mks1p from Grr1p-dependent Degradation in Wild-type Cells

We previously suggested that the form of Mks1p that inhibits the RTG pathway is a hyperphosphorylated species complexed with the 14-3-3 proteins, Bmh1p and Bmh2p (Liu *et al.*, 2003). That conclusion was based in part on the analysis of a dominant-negative *BMH1* mutant, *BMH1-1*, which shows a high level of *CIT2* expression and loss of glutamate

auxotrophy in *rtg2*Δ cells due to depletion of a functional Bmh2p pool. Bmh1p is the major isoform (Gelperin *et al.*, 1995), and Bmh1p and Bmh2p can function as homo- or heterodimers (Chaudhri *et al.*, 2003). Moreover, we also observed that the steady state level of Mks1p is substantially lower in *bmh1/2* mutant cells than in wild-type cells. These findings raise the possibility that Mks1p is not bound to the 14-3-3 proteins in *BMH1-1* cells and is rapidly degraded, thereby activating the RTG pathway. To test this, we first compared the stability of Mks1p expressed under the control of the *ADH1* promoter in *rtg2*Δ cells expressing either wild-type *BMH1* or the *BMH1-1* dominant mutant allele. As shown in Figure 3A, Mks1p-HA is unstable in *rtg2*Δ *BMH1-1* cells but is relatively stable in the *rtg2*Δ *BMH1* wild-type control. Similar data were obtained using Mks1p-HA expressed from its own promoter (Figure 2F and Supplementary Figure S4). Consistent with increased Mks1p-HA degradation in *rtg2*Δ *BMH1-1* cells, the steady-state level of Mks1p expressed from its own promoter was reduced by >10-fold in *rtg2*Δ *BMH1-1* cells (Supplementary Figure S5).

Next, we determined whether the increased degradation rate of Mks1p-HA is due to its failure to interact with Bmh1–1p. We performed coimmunoprecipitation experiments from extracts of cells in which Mks1p-HA was coexpressed with either wild-type Bmh1p-myc or the Bmh1–1p-myc dominant mutant. Immunoprecipitation of these proteins with anti-myc antibody shows that mutant Bmh1–1p has lost the ability to interact with Mks1p (Figure 3B). We can exclude the possibility that failure of Mks1p to interact with Bmh1–1p is due to misfolding of Bmh1–1p, such that it cannot form a heterodimer with Bmh2p, because coimmunoprecipitation experiments indicate that Bmh1–1p interacts as well with Bmh2p as does wild-type Bmh1p (Supplementary Figure S6). Further evidence that Mks1p depletion accounts for the *rtg2*Δ bypass phenotype in *BMH1-1* cells is the findings that overexpression of Mks1p in those cells from either a centromeric or 2μ plasmid under the control of the *ADH1* promoter restores glutamate auxotrophy, indicating that the RTG pathway has been down-regulated (Figure 3C).

To determine the stability of Mks1p when bound to Rtg2p in *BMH1-1* cells, Rtg2p was overexpressed from a *TEF1* promoter. Under these conditions, Mks1p was now stable (Figure 3, compare panels A and D). These data are similar to the preceding observations that the Rtg2p-bound form of Mks1p was also stable in *GRR1* dominant mutant cells (Figure 2D). To show that wild-type Grr1p targets Mks1p for degradation in *BMH1-1* cells, we inactivated *GRR1* in *rtg2*Δ cells expressing the dominant *BMH1-1* allele from a centromeric plasmid. Mks1p was now stable in those cells (Figure 3E).

To determine whether *GRR1* dependent instability of Mks1p in *rtg2*Δ *BMH1-1* cells is due to ubiquitination, Mks1p-HA expressed from the *ADH1* promoter was immunoprecipitated from a total cell extract using anti-HA antibody and Mks1p ubiquitination in the immunoprecipitate was probed with anti-ubiquitin antibody. Figure 3F shows that ubiquitination of Mks1p could be easily detected in *rtg2*Δ *BMH1-1* cells expressing Mks1p-HA and that ubiquitination was dependent on *GRR1*. To provide direct evidence that the Rtg2p- or Bmh1p- bound form of Mks1p is not a substrate for Grr1p-dependent ubiquitination, Bmh1p-myc was immunoprecipitated with anti-myc antibody and a 12xHis tagged Rtg2p was precipitated with Ni^{2+} beads from extracts of cells coexpressing Mks1p-HA. After a second precipitation with anti-HA antibody, the recovered Mks1p-HA was then examined for its ubiquitination state using anti-ubiquitin antibody. The Mks1p-HA recovered under these conditions was not ubiquitinated (Supplementary Figure S7), consistent with the notion that Rtg2p- or Bmh1p-bound form of Mks1p is not a substrate for Grr1p-dependent ubiquitination.

That *GRR1*-dependent degradation of Mks1p in *rtg2*Δ cells expressing the dominant *BMH1-1* mutant allele underlies the *rtg2*Δ bypass phenotype is evident from the findings that expression of the mutant *BMH1-1* allele, but not wild-type *BMH1*, in *rtg2*Δ cells results in increased *CIT2-lacZ* expression that is dependent on *GRR1* (Figure 3G). Together, these data suggest that Mks1p not bound to either Bmh1/2p or to Rtg2p is targeted by Grr1p for degradation. As expected, mutations in two other SCF^{Grr1} components, Cdc53p and Skp1p (Skowyra *et al.*, 1997; Patton *et al.*, 1998), also block *BMH1-1* induced degradation of Mks1p in *rtg2*Δ cells (Supplementary Figure S8).

*Functional Proteasomes Are Required for the rtg2*Δ *Bypass Phenotype in BMH1-1 and GRR1-1 Cells*

To demonstrate that the instability of Mks1p in *GRR1-1* and *BMH1-1* mutant cells is proteasome dependent, we treated cells with the proteasome inhibitor, MG132 (Lee and Goldberg, 1996). To facilitate uptake of MG132 into cells, an *erg6*Δ mutation was also introduced into *rtg2*Δ *GRR1-1* and *rtg2*Δ *BMH1-1* mutant strains. The resultant *rtg2*Δ *GRR1-1 erg6*Δ and *rtg2*Δ *BMH1-1 erg6*Δ cells were treated with or without 50 μM MG132 for 90 min before the cycloheximide chase. The instability of Mks1p expressed from the *ADH1* promoter in *rtg2*Δ *GRR1-1 erg6*Δ and *rtg2*Δ *BMH1-1 erg6*Δ cells was blocked in cells treated with MG132 (Figure 4A), indicating that Mks1p instability is dependent on functional proteasomes. To determine whether proteasomes are required for *rtg2*Δ bypass phenotype in *GRR1-1* and *BMH1-1* cells, *CIT2-lacZ* expression was examined in cells treated with or without MG132. The addition of MG132 had little effect on basal level of *CIT2-lacZ* expression in *erg6*Δ and *rtg2*Δ *erg6*Δ cells (Figure 4B). However, the high level of *CIT2-lacZ* expression observed in *rtg2*Δ *GRR1-1 erg6*Δ and *rtg2*Δ *BMH1-1 erg6*Δ cells was largely reversed by MG132. Thus the *rtg2*Δ bypass phenotypes of *BMH1-1* and *GRR1-1* mutant cells can be accounted for by proteasome-dependent degradation of Mks1p.

An Acidic Domain Region of Mks1p Contains the Degron Sequence

We next wanted to identify the domain of Mks1p conferring its Grr1p-dependent instability. Taking advantage of the robust degradation of Mks1p in *rtg2*Δ *GRR1-1* cells, we examined the stability of a series of Mks1p fragments (Figure 5A) with a 3xHA tag at their C-terminus expressed from centromeric plasmids under the control of the *ADH1* promoter. Figure 5B shows that an N-terminal Mks1p fragment (1–346) is unstable, whereas the shorter N-terminal fragments,1–304 and 1–275, are stable. The C-terminal fragment 276–584 and a shorter C-terminal fragment, 291–584, are also unstable in *rtg2*Δ *GRR1-1* cells, but further truncation to 305–584 results in a stable fragment. These results, summarized in Figure 5A, suggest that an acidic amino acid-rich region of Mks1p located between residues 291 and 346 is required to confer Mks1p instability in *rtg2*Δ *GRR1-1* cells. That fragment alone is unstable, and deleting a stretch of residues rich in acidic amino acids from 292 to 325 (Figure 4A) from full-length Mks1p to yield Mks1p (Δ292–325) confers stability to the protein. This acidic domain region of Mks1p is also required for wild-type *GRR1*-dependent Mks1p degradation in *rtg2*Δ *BMH1-1* cells (unpublished data). Sequence alignment of Mks1p from eight fungal species reveals five blocks of sequence conservation, shown

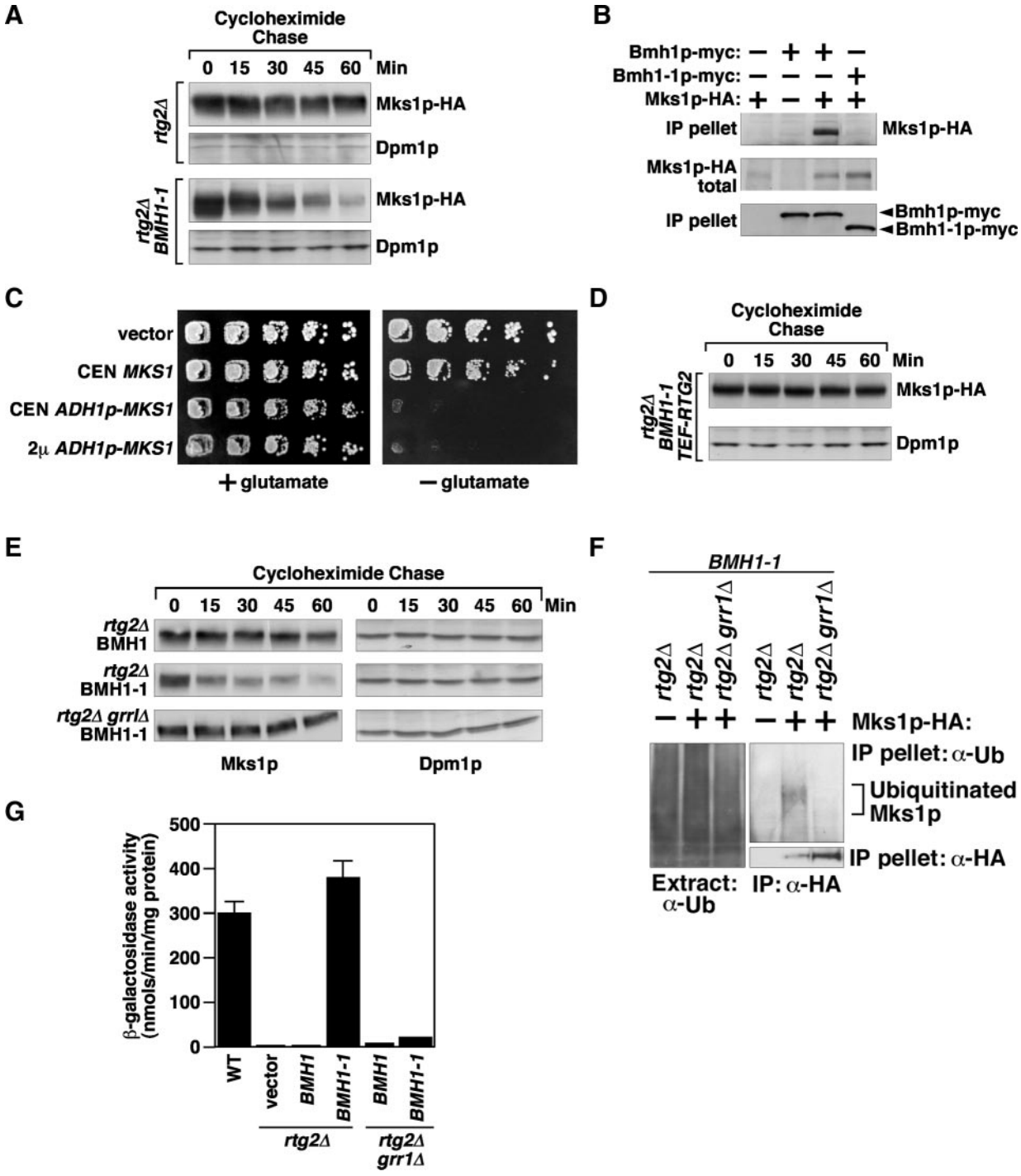

Figure 3. *GRR1*-dependent Mks1p instability in *rtg2*Δ *BMH1-1* cells. (A) Mks1p is unstable in *rtg2*Δ *BMH1-1* cells. Mks1p stability was examined in *rtg2*Δ or *rtg2*Δ *BMH1-1* cells expressing Mks1p under the control of the *ADH1* promoter using cycloheximide chase assay. (B) Mks1p-HA interacts with wild-type Bmh1p, but not mutant Bmh1–1p. Cell extracts of *bmh1*Δ *bmh2*Δ *mks1*Δ cells expressing the tagged proteins as indicated were obtained for immunoprecipitation. Anti-myc antibody was used to precipitate Bmh1p-myc and Bmh1–1p-myc. (C) Overexpression of *MKS1* reverses the *rtg2*Δ bypass phenotype of the *BMH1-1* mutation. *rtg2*Δ *BMH1-1* cells expressing different levels of *MKS1* were assayed for glutamate auxotrophy phenotype as described for Figure 2E. (D) Rtg2p protects Mks1p from degradation in *BMH1-1* cells. Mks1p stability was examined in *rtg2*Δ *BMH1-1* cells transformed with an integrated copy of *RTG2* under the control of *TEF1* promoter. (E) A *grr1*Δ mutation leads to Mks1p stability in *rtg2*Δ cells expressing *BMH1-1*. Mks1p stability was examined in *rtg2*Δ or *rtg2*Δ *grr1*Δ cells expressing wild-type *BMH1* or *BMH1-1* from a centromeric plasmid. (F) A *grr1*Δ mutation abolishes ubiquitination of Mks1p expressed from the *ADH1* promoter in *rtg2*Δ cells expressing the mutant Bmh1–1p. Total cell extracts were prepared from cells as indicated expressing Mks1p-HA under the control of the *ADH1* promoter. Mks1p-HA was immunoprecipitated using anti-HA antibody conjugated agarose beads. Anti-ubiquitin antibody was used to detect Mks1p ubiquitination. (G) A *grr1*Δ mutation reverses *BMH1-1*-induced *CIT2-lacZ* expression in *rtg2*Δ cells.

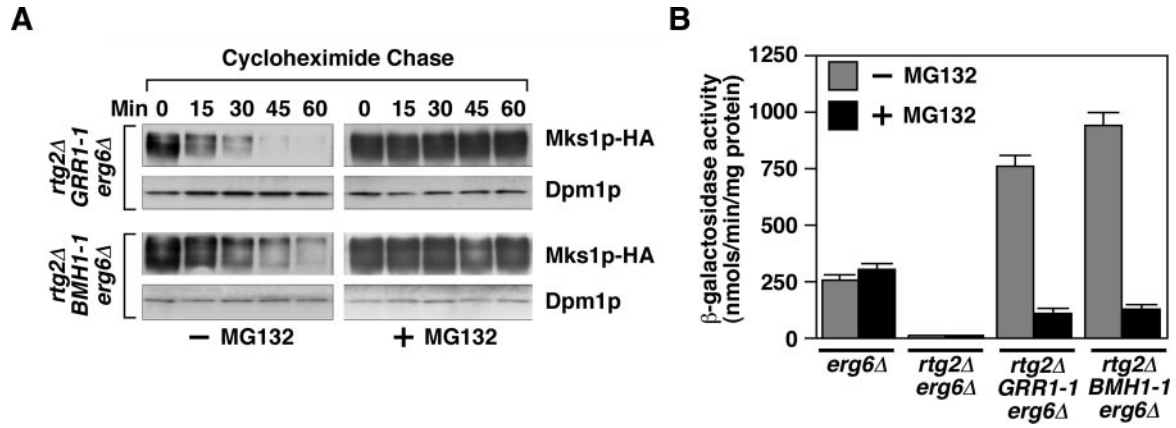

Figure 4. Functional proteasome is required for *rtg2*Δ bypass phenotype in *GRR1-1* and *BMH1-1* cells. (A) Mks1p is stabilized by MG132. Mks1p stability was examined in cells as indicated treated with or without MG132 (50 μM). Cells were grown in YNBcasD medium. (B) MG132 inhibits *CIT2-lacZ* expression in *GRR1-1* and *BMH1-1* cells. Indicated strains were grown in YNBcasD medium with or without MG132.

diagrammatically in Figure 5C, and in complete form in Supplementary Figure S9. Block II largely overlaps with the Mks1p acidic domain degron region (residues 291–346), underlying the importance of acidic domain region for Mks1p function.

To show that the acidic domain region of Mks1p is indeed a Grr1p degradation signal, we constructed a fusion protein containing residues 287–346 of Mks1p fused to the C-terminus of *Schistosoma japonicum* glutathione *S*-transferase (GST) and expressed this construct in *rtg2*Δ and *rtg2*Δ *grr1*Δ cells. In cells expressing wild-type Grr1p, the GST-Mks1p (287–346) fusion protein, by contrast to GST alone, is unstable over a 60-min cycloheximide chase, and this instability is dependent on wild-type Grr1p (Figure 5D). These data indicate that the acidic domain region of Mks1p contains the Mks1p degron sequence and that this degron is portable.

The Acidic Domain Region of Mks1p Is Required for Interaction with Rtg2p and Grr1p, but not with Bmh1p

Previous studies have shown that an Mks1p C-terminal fragment, 276–584, which includes the acidic domain, binds to Rtg2p (Liu *et al.*, 2003). The finding that Mks1p is stable when bound to Rtg2p, even in *GRR1* dominant mutant cells, raises the possibility that the acidic domain is not only a recognition site for Grr1p, but is also important for the binding of Mks1p to Rtg2p. If so, the acidic domain could be inaccessible to Grr1p in an Mks1p-Rtg2p complex. Conversely, the acidic domain may be less important for interaction of Mks1p with Bmh1/2p. These considerations would explain why Mks1p is insensitive to degradation when bound to Rtg2p but not to Bmh1/2p in cells expressing the dominant *GRR1* alleles.

To test these various possibilities, we asked whether the acidic domain region is important for binding to Grr1p, to the dominant mutant form of the protein, Grr1–1p, as well as to Rtg2p and Bmh1p. First, coimmunoprecipitation experiments were carried out with extracts of cells expressing HA-tagged Mks1p or its acidic domain deletion mutant, Mks1p (ΔAD) (Δ292–325), both under the control of *GAL1* promoter. Those same cells were also expressing either myc-tagged wild-type Grr1p, or mutant Grr1–1p, also under the control of *GAL1* promoter. Extracts were immunoprecipitated with anti-myc antibody and then probed by Western blotting for the various HA-tagged Mks1p derivatives. These experiments show that, in contrast to wild-type Mks1p-HA, the acidic domain deletion mutant, Mks1p (ΔAD)-HA, fails to interact with either Grr1p-myc or Grr1–1p-myc (Figure 6A).

Similar coimmunoprecipitation experiments were carried out to assess the role of the Mks1p acidic domain region in binding to Rtg2p (Figure 6B) and Bmh1p (Figure 6C). Figure 6B shows that wild-type Mks1p interacts with Rtg2p, whereas Mks1p (ΔAD) binds poorly to Rtg2p. These results suggest that Rtg2p protects Mks1p from degradation by blocking the binding site for Grr1p and Grr1–1p. In contrast, Bmh1p interacts with Mks1p (ΔAD) as well it does with wild-type Mks1p (Figure 6C). Previously, we showed that Bmh1p interacts with an N-terminal fragment of Mks1p, (1–346), but not with an overlapping C-terminal fragment (276–584; Liu *et al.*, 2003). 14-3-3 proteins usually bind to RxxpS/pT and RxxxpS/pT motifs (Yaffe *et al.*, 1997), and five such sites are located within residues 1–346 of Mks1p. However, mutation of the S/T residues in all five of those sites to alanines in the full-length protein does not affect the interaction of Mks1p with Bmh1p (Figure 6C) nor does it affect Mks1p functionality (unpublished data). Importantly, the Mks1p fragment 1–275 interacts well with Bmh1p. These data suggest that Bmh1p binds to Mks1p through the N-terminal 275 residues of Mks1p via novel binding sites.

To show that the acidic domain region of Mks1p is sufficient for interaction with Grr1p, we fused Mks1p residues 291–346 to the amino terminus of GST, which was expressed together with myc-tagged wild-type Grr1p in *rtg2*Δ cells. Coimmunopreciptation experiments show that the Mks1p acidic domain-GST fusion protein interacts with wild-type myc-tagged Grr1p, whereas no interaction was observed with GST alone (Figure 6D). The GST-Mks1p (291–346) fusion protein migrates as a single band on SDS-PAGE gels, and its mobility is unaffected by phosphatase treatment (unpublished data), suggesting that it is not phosphorylated. To provide additional support for the notion that the acidic domain region rather than phosphorylation is an important determinant for the interaction of Mks1p with Grr1p, we translated Mks1p in vitro and examined its interaction with a GST-Grr1p fusion protein expressed and purified from bacteria. Figure 6E shows that Grr1p tagged with GST, but not GST alone, interacts with in vitro-translated Mks1p, whereas the binding of the Mks1p (ΔAD) mutant protein is

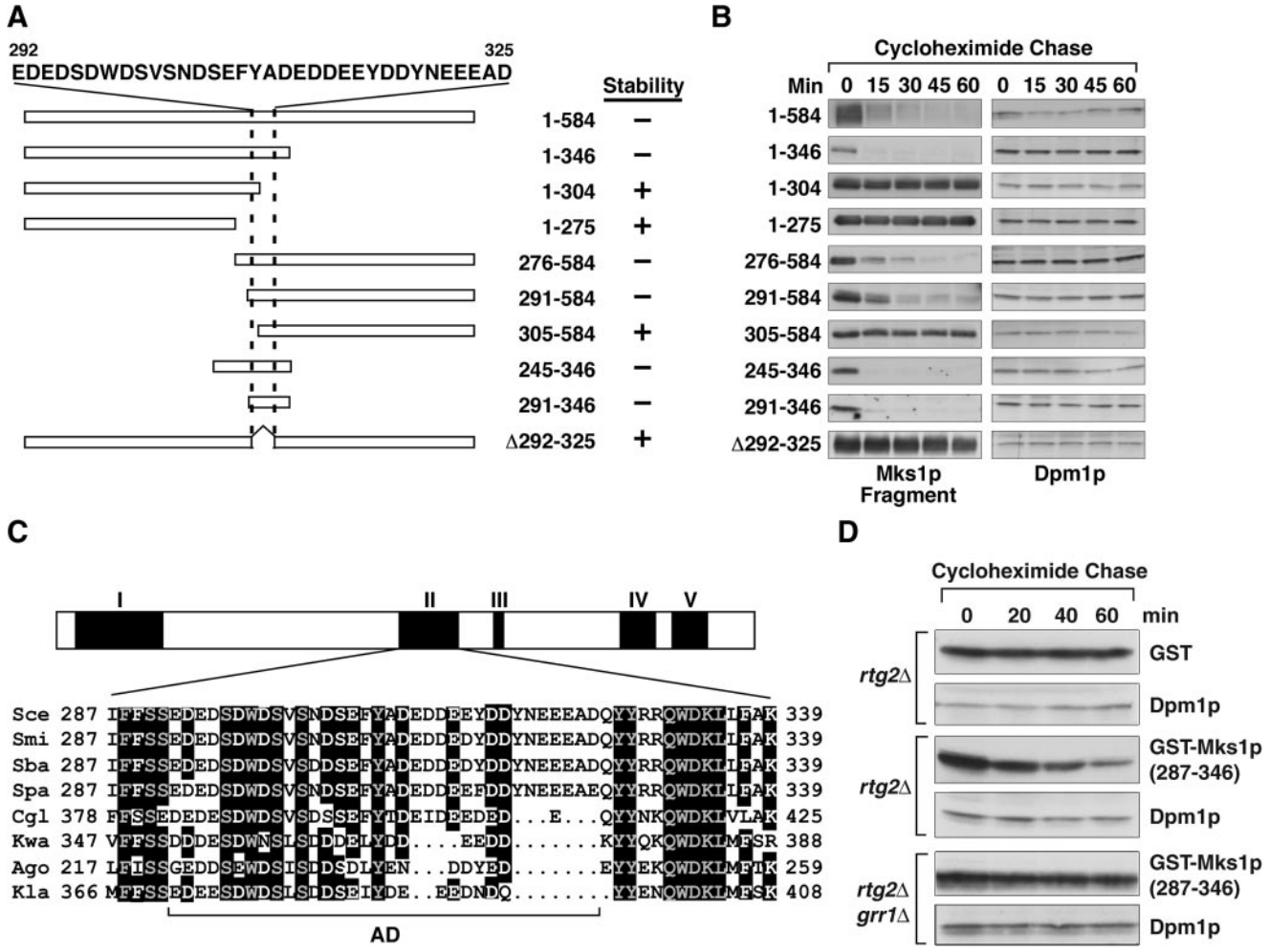

Figure 5. An acidic domain region of Mks1p contains the portable degron sequence. (A) Diagrammatic representation of constructs expressing various fragments of Mks1p. Numbers indicate the Mks1p residues expressed. Two dashed lines indicate a stretch of acidic amino acid-rich residues of Mks1p. (B) Residues 291–346 of Mks1p are required to confer Mks1p instability. The stability of various Mks1p constructs in *rtg2*Δ *GRR1-1* cells was determined using the cycloheximide chase assay. (C) A diagrammatic representation of sequence conservation of Mks1p from eight fungal species. Five conserved sequence blocks, I–V, are indicated, with Block II shown in detail. AD indicates a conserved acidic amino acid-rich subdomain. Sce, *S. cerevisiae*; Spa, *S. paradoxus*; Smi, *S. mikatae*; Sba, *S. bayanus*; Cgl, *Candida glabrata*; Kla, *Kluyveromyces lactis*; Kwa, *Kluyveromyces waltii*; Ago; *Ashbya gossypii*. (D) Mks1p residues 287–346 confer Grr1p-dependent instability to GST. GST or GST-Mks1p (287–346) fusion protein expressed in cells as indicated were examined for stability.

reduced by 10-fold (Figure 6E). Moreover, treatment of the in vitro-translated Mks1p with lambda phosphatase did not affect its mobility on SDS-PAGE or its ability to interact with Grr1p (Figure 6F). Taken together, these data suggest that the acidic domain region of Mks1p is both necessary and sufficient for interaction with Grr1p and that Mks1p phosphorylation does not play an essential role in recognition by Grr1p.

GRR1 Contributes to the Regulation of the RTG Pathway

To demonstrate that wild-type Grr1p plays a role in the regulation of the RTG pathway, we inactivated the wild-type *GRR1* allele and determined the effect on both basal and induced expression of *CIT2*. These different levels of *CIT2* expression are readily accomplished by growing cells in rich medium, in which there is a basal level of *CIT2* expression, or in minimal medium in which *CIT2* expression is highly induced (Liu and Butow 1999). Inactivation of *GRR1*, in contrast, has no effect on *CIT2* basal expression, but it markedly inhibited the induction of *CIT2* expression by growth of cells in minimal medium (Figure 7A). To provide additional support for Grr1p regulation of the RTG pathway via the regulation of Mks1p stability, we assayed for Mks1p ubiquitination in wild-type cells similar to the experiments described in Figure 3F. Figure 7B shows that Mks1p-HA expressed under the control of its own promoter is ubiquitinated in-wild type *GRR1* cells, but not in *grr1*Δ cells. Together with the previous observations that Mks1p exerts its highest inhibitory effect on the RTG pathway in the absence of Rtg2p (Dilova *et al.*, 2002; Sekito *et al.*, 2002), it is unlikely that Mks1p degradation in cells carrying an *rtg2*Δ mutation shown in Figures 2C and 3A is due to mis-folding of the protein. Furthermore, the constitutive high level expression of *CIT2* observed in *mks1*Δ cells (Sekito *et al.*, 2002) is not reduced by the introduction of a *grr1*Δ mutation (unpublished data), indicating that *MKS1* is epistatic to *GRR1*.

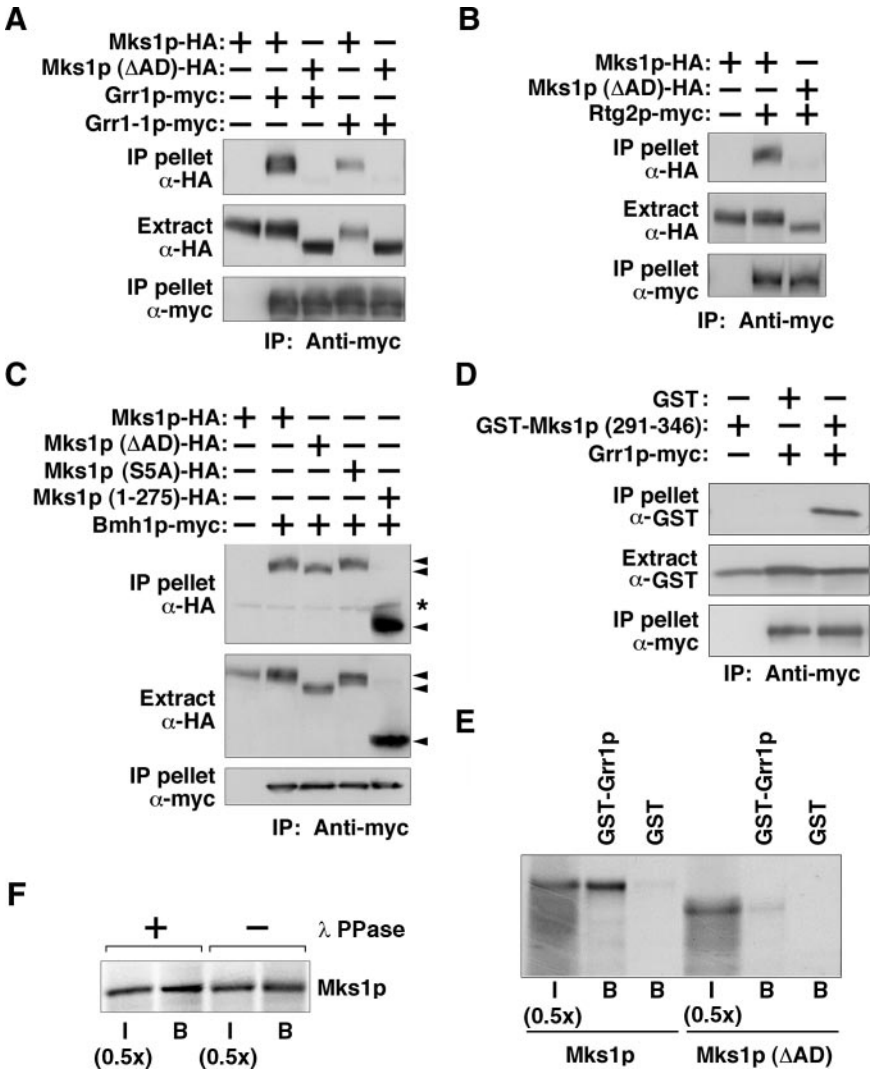

Figure 6. The Mks1p acidic domain (AD) is required for interaction with Grr1p and Rtg2p, but not Bmh1p. (A) The acidic domain of Mks1p is required for interaction with both wild-type Grr1p and with mutant Grr1–1p. *rtg2*Δ *erg6*Δ cells expressing HA- or myc-tagged proteins as indicated were grown in SCRaffGal medium. Coimmunoprecipitation experiments were carried out. (B) The acidic domain of Mks1p is required for Mks1p interaction with Rtg2p. Total cell extracts from *mks1*Δ cells expressing tagged proteins as indicated were subject to a coimmunoprecipitation assay. (C) Interaction between Bmh1p-myc and HA-tagged Mks1p. Total cell extracts from *bmh1*Δ *bmh2*Δ *mks1*Δ cells expressing tagged proteins as indicated were subject to a coimmunoprecipitation assay. "*" indicates the heavy chain of anti-myc antibody used for immunoprecipitation. (D) Mks1p acidic domain region is sufficient for interaction with Grr1p. Total cell extracts from *rtg2*Δ *erg6*Δ cells expressing tagged proteins as indicated were subject to a coimmunoprecipitation assay. GST also contains 48 amino acids at its C-terminus translated from an HA3 coding sequence and the polylinker, so its mobility is the same as the GST-Mks1p (291–346) fusion protein. Ub, ubiquitin. (E) In vitro-translated Mks1p interacts with Grr1p. Mks1p and Mks1p (ΔAD) were translated in vitro and labeled with ^{35}S-methionine as described in *Materials and Methods*.. The GST pulldown assay is described in *Materials and Methods*.. I, input fraction; B, glutathione sepharose beads bound fraction. (F) Interaction between GST-Grr1p and protein phosphatase treated Mks1p. In vitro-translated, ^{35}S-labeled Mks1p was treated with or without lambda protein phosphates (λ PPase) as indicated and subjected to a GST pulldown assay as described in E.

We have routinely observed that the glutamate auxotrophy phenotype of *rtg2*Δ cells is somewhat leaky (Figure 2E). If Grr1p contributes to the positive regulation of the RTG pathway, as suggested from the data above, then inactivation of *GRR1* should exacerbate the glutamate auxotrophy phenotype of *rtg2*Δ cells. This is indeed the case because *grr1*Δ and *rtg2*Δ mutations have additive effects on reducing cell growth on minimal medium without glutamate (Figure 7C). Taken together, we conclude that Grr1p is a positive regulator of the RTG pathway.

DISCUSSION

Regulation of the RTG pathway is achieved in part by factors that interact with Mks1p to modulate its activity. When bound to the redundant 14-3-3 proteins, Bmh1p and Bmh2p, Mks1p negatively regulates the RTG pathway; when bound to Rtg2p, Mks1p is inactive, resulting in high levels of *RTG* target gene expression. The present study reveals a third mode of regulation of Mks1p, whereby its abundance is controlled by SCF^{Grr1} E3 ubiquitin ligase-dependent polyubiquitination and degradation. This feature of Mks1p regulation appears to be important for the induction of *CIT2*, rather than for its basal level of expression. Our studies further suggest that the form of Mks1p targeted for degradation by wild-type Grr1p is not bound to either Rtg2p or Bmh1/2p. Why do cells need Grr1p as an additional modulator of Mks1p activity? The interaction of Mks1p with Rtg2p and Bmh1/2p is dynamic, switching the RTG pathway on and off depending on whether Mks1p is bound to Rtg2p or to Bmh1/2p (Liu *et al.*, 2003). We suggest that the efficiency of this switch would be enhanced, as summarized in the model depicted in Figure 8, by maintaining a low level of unbound Mks1p. In accordance with previous results (Liu *et al.*, 2003), we propose that a critical feature of Mks1p regulation is the two state interaction of Mks1p with either Bmh1/2p, which down-regulates the pathway by inhibiting the Rtg1/3p complex, or with Rtg2p, which prevents Mks1p from interacting with Bmh1/2p, thus activating the pathway. Mks1p not bound to either Rtg2p or to Bmh1/2p is proposed to be the preferred substrate for wild-type Grr1p, targeting Mks1p for degradation via the proteasome by polyubiquitination. This step would tighten control of the Mks1p on-off switch by keeping the unbound Mks1p pool low. We suggest that the Grr1p dominant mutants, by gaining one or two positive charges in the substrate binding surface, have a higher affinity for the acidic domain of Mks1p, allowing the mutant proteins access to Mks1p while

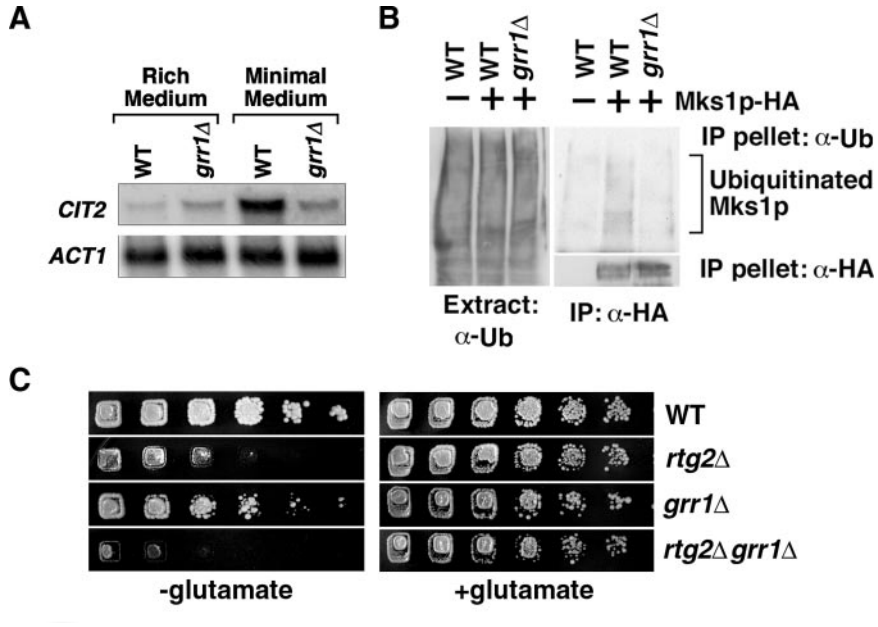

Figure 7. (A) A *grr1*Δ mutation blocks full induction of *CIT2* expression. Wild-type and *grr1*Δ cells were grown in rich YPD or minimal YNBD medium to midlog phase. Total mRNA was prepared and *CIT2* mRNA levels were determined by Northern blot analysis. *ACT1* was used as loading control. (B) A *grr1*Δ mutation blocks ubiquitination of Mks1p expressed from its own promoter. Ubiquitination of Mks1p was determined as described in Figure 3F. (C) The *grr1*Δ and *rtg2*Δ mutations additively reduce cell growth on medium lacking glutamate. Serial dilutions (5×) of cultures were prepared and cells were spotted onto solid YNBD medium with or without 0.02% glutamate. Cells were grown for 3–4 d at 30°C.

still bound to Bmh1/2p. However, the acidic domain is required for binding of Mks1p to Rtg2p, but not to Bmh1/2p, and thus may not be accessible to the dominant Grr1p mutants in the Mks1p-Rtg2p complex.

The dominant *GRR1* mutations, which lead to Rtg2p-independent activation of the RTG pathway, represent, to our knowledge, the first gain-of-function mutations described for a SCF E3 ubiquitin ligase. All of the independent, dominant *GRR1* mutants we identified have a net gain of one or two positive charges in the concave surface of the Grr1p LRR domain. The SCF E3 ubiquitin ligase family usually targets phosphorylated substrates, consistent with the modeled structure of the LRR domain in which there is a high density of positively charged residues on the concave, substrate-binding surface (Hsiung *et al.*, 2001). It has been shown that mutations of these basic residues to neutral or acidic amino acids results in an inability of the mutant protein to interact with one Grr1p substrate, Cln2p, resulting in its stabilization (Hsiung *et al.*, 2001). Our results lend further support to the view that the concave surface of LRR is the binding site for its substrates, likely through electrostatic interactions.

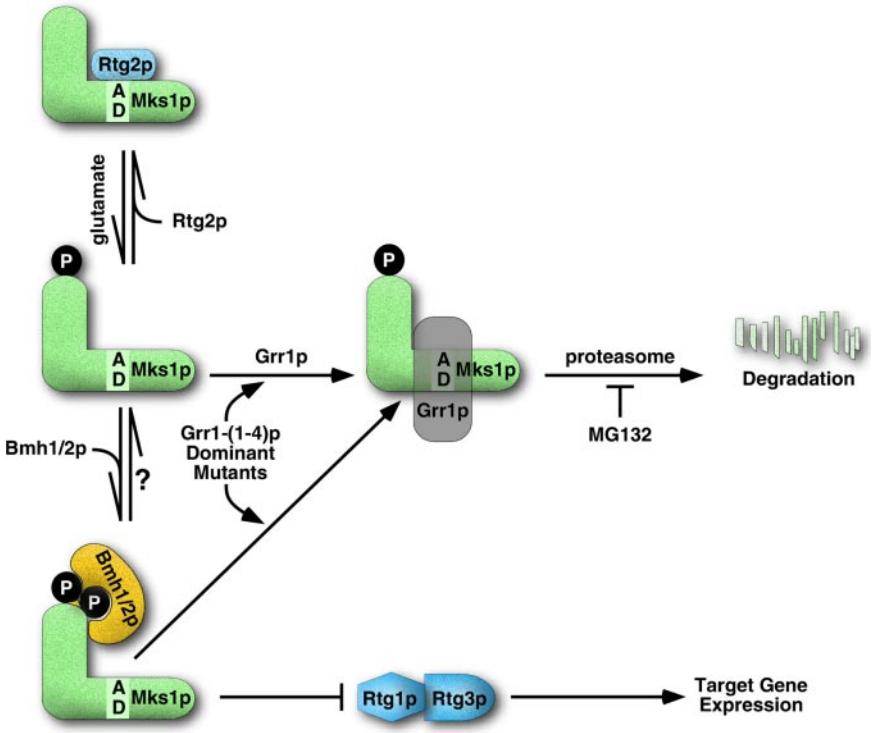

Figure 8. A model for regulation of the RTG pathway. Regulation relies on the dynamic interactions of Mks1p with Rtg2p and Bmh1/2p. When the RTG pathway is ON, Rtg2p binds to and inactivates Mks1p, which is hypophosphorylated. When the RTG pathway is OFF, Mks1p is released from Rtg2p, becomes hyperphosphorylated, and interacts with Bmh1/2p. The free Mks1p, unbound to either Rtg2p or Bmh1/2p, is degraded through SCF^{Grr1} dependent ubiquitination and degradation, which would keep free Mks1p level low to tighten control of Mks1p on-off switch and allow full activation of the RTG pathway. The acidic domain region in the central portion of Mks1p is Grr1p recognition motif. Dominant mutations on the concave surface of the Grr1p leucine-rich repeat domain allow Grr1p access to the acidic domain of Mks1p in the presence of Bmh1/2p, resulting Mks1p degradation.

The acidic domain region of Mks1p is both necessary and sufficient for interaction with Grr1p and is a novel feature of the recognition of a substrate by an E3 ubiquitin ligase. Although SCF E3 ubiquitin ligases are generally believed to recognize multiply phosphorylated substrates, for example, Sic1p, the target of Cdc4p (Verma *et al.*, 1997), and Cln2p, a target of Grr1p (Lanker *et al.*, 1996), we observed an acidic domain-dependent interaction of Grr1p with Mks1p in which Mks1p was synthesized in an unphosphorylated form by in vitro translation. However, we cannot exclude the possibility that Mks1p phosphorylation may play some role in modulating the efficiency by which Mks1p is recognized by Grr1p. A plausible explanation for the *GRR1* dominant mutant phenotype is that the gain of positive charges in the LRR domain increases the affinity of the interaction between Grr1p and the acidic domain of Mks1p, thereby allowing Mks1p to be targeted for degradation when bound to Bmh1/2p.

The finding that the acidic domain of Mks1p is also required for its interaction with Rtg2p provides an explanation for the resistance of the Rtg2p-bound form of Mks1p to degradation in the *GRR1* wild-type or dominant mutant background: in the Mks1p-Rtg2p complex, the acidic domain would likely be occluded from interaction with Grr1p. By contrast, the acidic domain is not required for Mks1p binding to Bmh1/2p, and thus when bound to Bmh1/2p it would still be available for interaction with a Grr1p mutant with a presumed higher affinity for the acidic domain. The Bmh1/2p-bound form of Mks1p may nevertheless result in some physical hindrance to the accessibility of the Mks1p acidic domain, because Bmh1/2p protects Mks1p from being targeted for degradation by wild-type Grr1p. Thus one important function of Bmh1/2p because negative regulators of the RTG pathway would be to protect Mks1p from being targeted for degradation by wild-type Grr1p. Precisely how retrograde signals are translated to switch Mks1p binding between Rtg2p to Bmh1/2p is presently unclear. However, we have observed that the dissociation of Mks1p from Rtg2p requires ATP hydrolysis (our unpublished data), suggesting the possibility that signaling may also involve some monitoring of ATP/ADP levels.

Inhibition of TOR kinase activity by rapamycin treatment activates the RTG pathway (Komeili *et al.*, 2000; Shamji *et al.*, 2000). There exist two TOR kinase complexes, TORC1 and TORC2, both of which contain Lst8p (Loewith *et al.*, 2002; Wedaman *et al.*, 2003). Isolation and characterization of *lst8* mutations which bypass the *rtg2*Δ mutation suggest that TOR kinases play a role in regulation of the RTG pathway (Chen and Kaiser, 2003; Liu *et al.*, 2003). The findings presented here raise questions whether activation of the RTG pathway by rapamycin treatment is due to increased Mks1p instability. However, we found that rapamycin fails to increase Mks1p turnover in either wild-type cells or in *rtg2*Δ mutant cells (unpublished data), indicating that Mks1p degradation represents a TOR-independent process in the regulation of the RTG pathway. Because activation of the RTG pathway by rapamycin treatment requires Rtg2p (Komeili *et al.*, 2000 and our unpublished observations), we believe that increased Mks1p binding to Rtg2p is likely to be the mechanism of rapamycin-induced activation of the RTG pathway.

14-3-3 proteins are involved in multiple physiological pathways and usually bind their substrates through phospho-serine or threonine at Rxx(x)pS/pT motifs (Yaffe *et al.*, 1997). Similar to the protection effect of Bmh1/2p on Mks1p, a 14-3-3 protein protects the tumor suppressor p53 from degradation by a E3 ubiquitin ligase, Mdm2 (Yang *et al.*, 2003). Mks1p contains five consensus RxxpS/pT sites within an N-terminal region (1–275), which we have shown here binds to Bmh1/2p. We have mutated all five of those sites, but have not observed any effect on Bmh1p binding (Figure 6C). This suggests that Bmh1/2p may recognize novel sites in the N-terminal domain of Mks1p.

Grr1p is known to regulate the stability of the G1 cyclins, Cln1 and Cln2, and the putative Cdc42p regulator, Gic2p (Deshaies, 1999). Grr1p is also required for glucose induction of several hexose transporters, including Hxt1p, by increasing degradation of a negative regulator Mth1p (Li and Johnston, 1997; Flick *et al.*, 2003). Grr1p has also been reported to be required for amino acid induced expression of several amino acid permeases, including Agp1p, and a peptide transporter, Ptr2p (Bernard and Andre, 2001). We have examined all four *GRR1* dominant mutant alleles for their potential effects on expression of *AGP1*, *HXT1*, and *PTR2* and no effects have been found thus far (our unpublished data). We have also failed to see an effect of the *GRR1-1/2* mutations on Cln2 stability (Supplementary Figure S3). These results suggest that Grr1p utilizes different sites to interact with regulators in different pathways and are consistent with the finding that neutralization of positively charged residues in the concave surface of LRR of Grr1p results in defects in Cln2 degradation but has no effect on transcription of *HXT1* and *AGP1* (Hsiung *et al.*, 2001).

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