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Award Number: W81XWH-05-1-0140

TITLE: Fission Yeast Model Study for Dissection of TSC Pathway

PRINCIPAL INVESTIGATOR: Tomohiro Matsumoto, Ph.D.

CONTRACTING ORGANIZATION: Kyoto University
Kyoto, Japan 606-8501

REPORT DATE: April 2010

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE April 2010		2. REPORT TYPE Final		3. DATES COVERED 30 December 2004 – 29 March 2010	
4. TITLE AND SUBTITLE Fission Yeast Model Study for Dissection of TSC Pathway				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0140	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Tomohiro Matsumoto E-Mail: tmatsumo@house.rbc.kyoto-u.ac.jp				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Kyoto University Kyoto, Japan 606-8501				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Tuberous sclerosis complex (TSC) is an autosomal dominant disorder associated with mutations in two different genes, TSC1 and TSC2, which cause benign tumors called hamartomas. In the fission yeast model study, we have demonstrated that a mutation in a gene encoding a !-subunit of a farnesyl transferase can suppress most of the phenotypes associated with deletion of tsc1+ or tsc2+. When a mutant of rhb1+ (homolog of human Rheb), which bypasses the requirement of protein farnesylation, was expressed, the cpp1-1 mutation could no longer suppress a tsc2-null strain. The result would suggest that a drug inhibiting a farnesyl transferase may be used for treatment of TSC. We have also generated two mutants, rhb1-DA4 and rhb1-DA8. In fission yeast, two events, induction of a meiosis initiating gene mei2+ and cell division without cell growth, are a typical response to nitrogen starvation. We have found that while amino acid uptake is prevented by both rhb1-DA4 and rhb1-DA8, the response to nitrogen starvation is prevented only by rhb1-DA4. We postulate that the signaling cascade may branch below Rhb1. If human Rheb GTPase works in a similar manner, the efficacy of Rapamycin, an anti-TSC drug targeting mTOR, may be limited.					
15. SUBJECT TERMS None provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	29	19b. TELEPHONE NUMBER (include area code)

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Introduction: Tuberous sclerosis complex (TSC) is an autosomal dominant disorder associated with mutations in two different genes, TSC1 and TSC2, which cause benign tumors called hamartomas. It is speculated that loss of TSC1/2 would result in constitutive activation of RHEB GTPase and its downstream elements mTOR, which, in turn, activates S6K and eIF-4E for protein synthesis. Rapamycin is an inhibitor of mTOR and is currently considered as a drug for treatment of TSC. If hamartomas is attributable solely to abnormal activation of mTOR via RHEB, rapamycin may be an effective drug to cope with TSC. On the other hand, if TSC1/2 cascade regulates a yet unidentified element in addition to RHEB and mTOR, the efficacy of rapamycin is probably limited.

The TSC1/2 signaling cascade is evolutionarily conserved from human through fission yeast. Loss of either Tsc1 or Tsc2 in fission yeast causes two defects; abnormal localization of an amino-acid permease and lack of induction of *sxa2+* gene upon nitrogen starvation. We speculate that a failure in nutrient sensing would be the primary cause of the two defects. However, the responsible downstream element of Tsc1/2, which regulates localization of the permease or gene expression of *sxa2+*, could be different/independent. Taking advantage of the power of genetics in the fission yeast model system, we will address the following questions;

Q1. Are all the phenotypes associated with loss of Tsc1/2 attributable to the constitutive activation of Rhb1 (fission yeast homolog of the human RHEB)?

Q2. Whom else does Tsc1/2 talk to in addition to Rhb1?

Specific Aims

Aim 1: We will generate a dominant active Rhb1 and express it in fission yeast. The phenotypes observed upon expression will be compared to those in *tsc1*- or *tsc2*-null yeast. We will also tag Rhb1 with HA epitope to facilitate biochemical analysis.

Aim 2: We will identify suppressor genes whose upregulation or downregulation rescues the phenotypes associated with loss of Tsc1/2.

Body: The original proposal was submitted with the following 9 tasks most of which were completed and published;

Task 1: To generate a dominant active Rhb1 (Month 1-8)
Mutagenesis of Rhb1 gene and cloning, expression in yeast
Evaluation of the phenotypes

Task 2: To tag Rhb1 with HA epitope (Month 9-18)
Cloning and expression of HA-tagged Rhb1
Biochemical analysis of HA-Rhb1

Task 3: To isolate extragenic suppressors (Month 1-6)
Mutagenesis and isolation of revertants
Basic genetic analysis (tetrad analysis and linkage analysis)

Task 4: To characterize extragenic suppressors (Month 7-18)
Determination of the locus- and phenotypic-specificity
Biochemical analysis of Rhb1 in extragenic suppressors
Data evaluation

Task 5: To clone the corresponding genes for extragenic suppressors (Month 19-24)
Transformation and isolation of the corresponding gene
Determination of sequencing

Task 6: To conclude study for extragenic suppressors (Month 25-30)
Additional genetic and biochemical analysis
Data evaluation
Preparation/submission of a manuscript

Task 7: To isolate multicopy suppressors (Month 13-20)
Transform *tsc1*-null and *tsc2*-null yeast
Isolate revertants
Recover plasmids to *E. coli*
Determine the structure of the plasmids
Identify the suppressor genes

Task 8: To characterize multicopy suppressors (Month 20-30)
Determination of the locus- and phenotypic-specificity
Biochemical analysis of Rhb1 in extragenic suppressors
Data evaluation

Task 9: To conclude study for multicopy suppressors (Month 31-36)
Additional genetic and biochemical analysis
Data evaluation
Preparation/submission of a manuscript

Tasks 1 and 2 were reported in Annual reports of 2006, 2007, 2008 and 2009 and published in *Genetics* 183(2), 517-527 (2009).

Tasks 3, 4, 5 and 6 were reported in Annual reports of 2007, 2008 and 2009. Some of the results were published in *Genetics* 173, 569-578 (2006). The results not published yet (reported in 2008 and 2009) will be published after additional experiments are completed.

Tasks 7 and 8 were reported in Annual reports of 2007 and 2008, but have not been published yet (Task 9). Additional biochemical studies to reveal an underlying mechanism of the suppression are necessary.

Key Research Accomplishments

This research project has yielded three highlights; 1) We have analyzed gene expression profile in a genome-wide scale and found that deletion of either *tsc1*⁺ or *tsc2*⁺ affects gene induction upon nitrogen starvation. Three hours after nitrogen depletion genes encoding permeases and genes required for meiosis are less induced. Under the same condition, retrotransposons, G1-cyclin (*pas1*⁺) and *inv1*⁺ are more induced. We have also demonstrated that a mutation (*cpp1-1*) in a gene encoding a β -subunit of a farnesyl transferase can suppress most of the phenotypes associated with deletion of *tsc1*⁺ or *tsc2*⁺. When a mutant of *rhb1*⁺ (homolog of human *Rheb*), which bypasses the requirement of protein farnesylation, was expressed, the *cpp1-1* mutation could no longer suppress, indicating that deficient farnesylation of Rhb1 contributes to the suppression. (*Genetics* 173, 569-578, 2006), 2) We have generated

two mutants, *rhb1-DA4* and *rhb1-DA8*, and characterized them genetically. V17A mutation within the G1 box defined for the ras-like GTPases was responsible for *rhb1-DA4*, and Q52R I76F within the switch II domain for *rhb1-DA8*. In fission yeast, two events, induction of a meiosis initiating gene *mei2*⁺ and cell division without cell growth, are a typical response to nitrogen starvation. Under nitrogen-rich conditions, Rheb stimulates Tor kinase, which, in turn, suppresses the response to nitrogen starvation. We have found that while amino acid uptake is prevented by both *rhb1-DA4* and *rhb1-DA8* in a dominant fashion, the response to nitrogen starvation is prevented only by *rhb1-DA4*. *rhb1-DA8* thereby allows genetic dissection of the Rheb-dependent signaling cascade. We postulate that the signaling cascade may branch below Rhb1 or Tor2 and regulate the amino acid uptake and response to nitrogen starvation independently (Genetics 183, 517-527, 2009), and finally 3) By performing a genetic screen for extragenic suppressors of a strain with deletion of *tsc2*⁺ (Tasks 3 and 4), we have isolated several loci in addition to the *cpp-1* locus. The corresponding genes (though yet to be identified at this stage) are our asset for the future study.

Reportable Outcomes (publication)

Nakase et al., Genetics 173, 569-578, 2006

Murai et al., Genetics 183, 517-527, 2009

(oral presentation)

Tomoka Murai, Yukiko Nakase, Yuji Chikashige, Chihiro Tsutsumi Yasushi Hiraoka, and Tomohiro Matsumot Distinctive responses to nitrogen starvation in the dominant active mutants of the fission yeast Rheb GTPase. The 5th International Fission Yeast Meeting, October, 2009 Tokyo

Title: Molecular and functional dissection of Rhb1 GTPase in fission yeast

Abstract: Signalling involving mTOR kinase is evolutionally conserved from yeast to human. In vertebrates, it regulates cell growth depending on the availability of nutrients, energy source, and growth factors. In fission yeast, a prototype of the TOR signaling system controls various biological responses to starvation for nitrogen. Just like the mTOR signaling system in higher eukaryotes, it consists of Tsc1/2 (a GTPase-activating protein complex), Rhb1 GTPase (homolog of RHEB) and Tor2 (homolog of mTOR).

Starvation for nitrogen induces adaptive events including among others, elevation of the uptake of amino-acids and induction of the *mei2* gene in fission yeast. Upon starvation, Tsc1/2 protein complex presumably converts Rhb1 GTPase into a GDP-bound (inactive) form. As a result, adaptive events to nitrogen starvation, which are suppressed under nitrogen-rich conditions, are induced. Tor2, a downstream element of Rhb1, is responsible for suppression of *mei2* gene under nitrogen-rich conditions. Upon starvation, Tor2-dependent suppression is relieved as Rhb1 GTPase is converted to an inactive form, which can no longer activate Tor2. In contrast, the underlying mechanism of the elevation of the uptake is not well understood. Nonetheless, both events are under control of Rhb1 GTPase as we previously showed that fission yeast strains lacking Tsc2 cannot induce either the elevation of uptake or expression of the *mei2* gene. In this study, we focus on the Rhb1 GTPase and attempt to understand how it signals to TOR as well as other components. Two mutants of *rhb1*⁺ (*rhb1-DA4* and *rhb1-DA8*) have been characterized. The mutation of *rhb1-DA4* was identified in the G1 box, a domain, which is important for GTP binding as well as its hydrolysis. A strain expressing *rhb1-DA4* in place of the wild type *rhb1*⁺ exhibits phenotypes similar to those of strains lacking Tsc2. It cannot induce either the elevation of the uptake of amino-acids or expression of the *mei2*⁺ gene after nitrogen starvation. The phenotypes and the mutation site suggest that Rhb1-DA4 might remain as a GTP-bound active form just like the oncogenic Ras GTPase.

Remarkably, the other mutant, *rhb1-DA8*, which has the responsible mutation around switch II domain (a region close to the effector loop), exhibits a phenotype different from that of *rhb1-DA4*. While it can normally induce expression of the *mei2⁺* gene in response to nitrogen starvation, it is unable to elevate uptake of amino-acids.

Tor2 has been the only known effector of Rhb1. The results of this study would suggest that Rhb1 GTPase may have at least two effectors, Tor2, which can be constitutively activated by Rhb1-DA4, but not by Rhb1-DA8, and the other one, which can be specifically activated by Rhb1-DA8 even after nitrogen starvation.

Personnel

- 1) Yukiko Nakase (Postdoctoral research fellow)
- 2) Tomoyo Oda (Technician)

Conclusion

The proposal was submitted with the following two specific aims: [Aim 1]: We will generate a dominant active Rhb1 and express it in fission yeast. The phenotypes observed upon expression will be compared to those in *tsc1-* or *tsc2-* null yeast. We will also tag Rhb1 with HA epitope to facilitate biochemical analysis and [Aim 2]: We will identify suppressor genes whose upregulation or downregulation rescues the phenotypes associated with loss of Tsc1/2.

For Aim1, we have generated two dominant active Rhb1 (DA4 and DA8) and shown that the *rhb1-DA4* mutant can phenocopy the *tsc1-* and *tsc2-2* null strains. We would therefore conclude that the major function of Tsc1/2 is to convert Rhb1-GTPase from a active to an inactive form. For Aim2, we have isolated several extragenic and multicopy suppressors. We have shown that one of them, *Cpp1*, is an enzyme to enhance the activity of Rhb1, demonstrating a new mechanism to regulate Rhb1.

In addition to these two results published, we have identified several new loci through genetic screens as a candidate of a downstream element of Tsc1/2.

References

- 1: European Chromosome 16 Tuberous Sclerosis Consortium, 1993 Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 75: 1305-1315.
- 2: van Slegtenhorst, M., R. de Hoogt, C. Hermans, M. Nellist, B. Janssen *et al.*, 1997 Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 277: 805-808.

Appendix

A Defect in Protein Farnesylation Suppresses a Loss of *Schizosaccharomyces pombe tsc2⁺*, a Homolog of the Human Gene Predisposing to Tuberous Sclerosis Complex

Yukiko Nakase,^{*,†} Keiko Fukuda,^{*,†} Yuji Chikashige,[‡] Chihiro Tsutsumi,[‡] Daisuke Morita,[§] Shinpei Kawamoto,[§] Mari Ohnuki,[§] Yasushi Hiraoka,[‡] and Tomohiro Matsumoto^{*,†,1}

^{*}Radiation Biology Center, Kyoto University, Yoshida-Konoe cho, Sakyo ku, Kyoto, Japan, 606-8501, [†]Graduate School of Biostudies, Kyoto University, Kitashirakawa-Oiwake cho, Sakyo ku, Kyoto, Japan, 606-8502, [‡]Department of Agriculture, Kyoto University, Kitashirakawa-Oiwake cho, Sakyo ku, Kyoto, Japan, 606-8502 and [§]Cell Biology Group, Kansai Advanced Research Center National Institute of Information and Communications Technology, Kobe, Hyogo, Japan, 651-2492

Manuscript received February 8, 2006
Accepted for publication March 31, 2006

ABSTRACT

Mutations in the human *Tsc1* and *Tsc2* genes predispose to tuberous sclerosis complex (TSC), a disorder characterized by the wide spread of benign tumors. Tsc1 and Tsc2 proteins form a complex and serve as a GTPase-activating protein (GAP) for Rheb, a GTPase regulating a downstream kinase, mTOR. The genome of *Schizosaccharomyces pombe* contains *tsc1⁺* and *tsc2⁺*, homologs of human *Tsc1* and *Tsc2*, respectively. In this study we analyzed the gene expression profile on a genomewide scale and found that deletion of either *tsc1⁺* or *tsc2⁺* affects gene induction upon nitrogen starvation. Three hours after nitrogen depletion genes encoding permeases and genes required for meiosis are less induced. Under the same condition, retrotransposons, G1-cyclin (*pas1⁺*), and *inv1⁺* are more induced. We also demonstrate that a mutation (*cpl1-1*) in a gene encoding a β -subunit of a farnesyltransferase can suppress most of the phenotypes associated with deletion of *tsc1⁺* or *tsc2⁺*. When a mutant of *rhb1⁺* (homolog of human *Rheb*), which bypasses the requirement of protein farnesylation, was expressed, the *cpl1-1* mutation could no longer suppress, indicating that deficient farnesylation of Rhb1 contributes to the suppression. On the basis of these results, we discuss TSC pathology and possible improvement in chemotherapy for TSC.

TUBEROUS sclerosis complex (TSC) is an autosomal dominant disorder characterized by the wide spread of benign tumors called hamartomas in different organs including the brain, eyes, heart, kidney, skin, and lungs (Kwiatkowski and Short 1994; Gomez 1995). Seizures and learning and behavioral problems, which are likely due to development of tumors in the brain, are also common in patients with TSC (Kwiatkowski and Short 1994; Gomez *et al.* 1999). Two human genes, *TSC1* and *TSC2*, are responsible for TSC (European Chromosome 16 Tuberous Sclerosis Consortium 1993; van Sleightenhorst *et al.* 1997), each of which encodes hamartin and tuberin, respectively. Inactivation of *TSC1* and *TSC2* causes phenotypes similar each other, suggesting that they might affect the same pathway. *TSC1* and *TSC2* form a heterodimer and the *TSC1*–*TSC2* interaction appears to be important for the stability of the two proteins (Li *et al.* 2004b). Therefore, *TSC1* and *TSC2* are generally considered as a complex (*TSC1/2*) with a single bio-

logical function, and understanding functions of the *TSC1/2* complex is clinically important.

Genetic studies in mammalian systems (Carbonara *et al.* 1994; Green *et al.* 1994a,b; Henske *et al.* 1996; Kwiatkowski *et al.* 2002) and *Drosophila* (Ito and Rubin 1999) have shown that *TSC1/2* functions to inhibit cell growth as well as cellular proliferation (Hengstschlager *et al.* 2001). Appearance of giant cells within hamartomas from TSC patients and the *gigas* phenotype in the fly mutant highlight the capability of *TSC1/2* in controlling cell size (Ito and Rubin 1999). Studies have shown that *TSC1/2* controls cell growth/proliferation by regulating the activity of a small GTPase, RHEB (Zhang *et al.* 2003). When the environment surrounding the cell is not favorable for growth/proliferation, *TSC1* and *TSC2*, which have a GTPase-activating protein (GAP) domain in their C-terminal region, convert RHEB into an inactive form. A kinase, mTOR is a target of RHEB and promotes protein synthesis when stimulated by RHEB GTPase (Manning and Cantley 2003; Li *et al.* 2004a,b; Pan *et al.* 2004; Long *et al.* 2005). It is postulated that formation of hamartomas in TSC is a result of abnormal regulation of RHEB GTPase. A loss of *TSC1/2* would allow constitutive activation of the GTPase as well as its target, mTOR.

¹Corresponding author: Radiation Biology Center, Kyoto University, Yoshida-Konoe cho, Sakyo Ku, Kyoto, Japan 606-8501.
E-mail: tmatsumo@house.rbc.kyoto-u.ac.jp

TABLE 1
Strains used in this study

Strain	Genotype	Source
SP6	<i>h⁻ leu1-32</i>	Laboratory stock
AE512	<i>h⁻ tsc2::ura4⁺ ura4-D18 leu1-32</i>	Laboratory stock
AE413	<i>h⁻ tsc1::ura4⁺ ura4-D18 leu1-32</i>	Laboratory stock
YKK25	<i>h⁻ tsc2::ura4⁺ ura4-D18 leu1-32 cpp1-1</i>	This work
YKK59	<i>h⁻ tsc1::ura4⁺ ura4-D18 leu1-32 cpp1-1</i>	This work
972h ⁻	<i>h⁻</i>	Laboratory stock
YKK55	<i>h⁻ ura4-D18 leu1-32 cpp1-1</i>	This work
SP740	<i>h⁻ ura4-D18 leu1-32</i>	Laboratory stock

Homologs of the mTOR kinase and RHEB can be found in lower eukaryotes. The genome of *Schizosaccharomyces pombe* contains two genes homologous to mTOR (*tor1⁺* and *tor2⁺*) and a gene homologous to RHEB, *rhl1⁺* (MACH *et al.* 2000). It also contains genes *tsc1⁺* and *tsc2⁺*, each of which corresponds to mammalian *TSC1* and *TSC2*, respectively (MATSUMOTO *et al.* 2002). Although the genome of *Saccharomyces cerevisiae* also encodes proteins homologous to mTOR (CAFFERKEY *et al.* 1994) and RHEB GTPase (URANO *et al.* 2000), it does not contain any obvious homologs to *TSC1/2*, suggesting that RHEB GTPase may be regulated by another mechanism.

In our previous study we showed that fission yeast strains lacking either *tsc1⁺* or *tsc2⁺* are viable in rich media, but exhibit several defects. First, deletion strains for *tsc1⁺* ($\Delta tsc1$) and *tsc2⁺* ($\Delta tsc2$) are defective in uptake of nutrients such as amino acids and adenine. Consistent with this defect, an amino acid permease, which is normally positioned on the plasma membrane, aggregates in the cytoplasm or is confined in vacuole-like structures in $\Delta tsc1$ and $\Delta tsc2$. Second, $\Delta tsc1$ and $\Delta tsc2$ are unable to induce the *sxa2⁺* gene, which is usually expressed upon stimulation by a mating-type pheromone, *P*factor, in starved *h⁻* cells (IMAI and YAMAMOTO 1994). On the basis of these phenotypes, we postulate that *tsc1⁺* and *tsc2⁺* are required for sensing/responding to starvation. We speculate that *S. pombe* *Tsc1/2* regulates *Tor1/2* via *Rhl1* and plays a role in sensing/responding to starvation. In this study we continued to take advantage of this simple and tractable system and attempted to dissect genetic pathways to interact with *Tsc1/2*. Through a genetic screen of extragenic suppressors of $\Delta tsc2$, we identified a gene, *cpp1⁺*, encoding a subunit of the enzyme required for protein farnesylation.

MATERIALS AND METHODS

Yeast strains, media, and transformation: The *S. pombe* strains used in this study are listed in Table 1. The yeast cells were grown in YEA and EMM with appropriate nutrient supplements as described previously (MORENO *et al.* 1991). All yeast transformations were carried out by lithium acetate methods (OKAZAKI *et al.* 1990; GIETZ *et al.* 1992).

Screen for an extragenic suppressor of $\Delta tsc2$: The reversion rate of the AE512 strain used for the screening was 1.25×10^{-6} . The spontaneous revertants were grown at 26° for 4 days on EMM medium with leucine at 40 µg/ml. Sixty-five revertants obtained through the primary screen were tested for their temperature sensitivity in the secondary screen. The revertants were replicated on two YEA plates and incubated at 26° (for 3 days) or 36° (for 2 days), respectively. Among the revertants isolated through the primary screen, 11 strains exhibited a temperature sensitivity for growth at 36°. Finally, the 11 revertants were further tested for their ability to induce *fnx1⁺* and *mei2⁺* upon nitrogen starvation by Northern analysis. Two revertants satisfied the final criterion and were further examined genetically.

Cloning of *cpp1⁺*: The *cpp1-1* mutant (YKK25) was transformed with an *S. pombe* genomic library containing partially digested *Sau3AI* DNA fragment constructed in a multicopy plasmid, pAL-KS (TANAKA *et al.* 2000). Plasmids were recovered from Ts⁺ Leu⁺ transformants and their nucleotide sequences were determined. BLAST search was performed for the obtained sequences, and the region covered by the inserted genomic sequence was determined.

Plasmid construction: Plasmid pREP41-cpp1 was constructed as follows. The *cpp1⁺* gene was amplified by PCR using the forward primer F-cpp1 [5'-CCCCCGTCGAC(*SalI*)GATGGATGAATTATCAGAAAC-3'] and the reverse primer R-cpp1 [5'-CCCCCGGATCC(*Bam*HI)TTAGAAATTTTGATGATTCTTG-3']. The resulting fragment was digested with *Bam*HI and *SalI* and then cloned into pREP41 (MAUNDRELL 1993). Plasmids pREP41-rhl1 and pREP81-rhl1 were constructed as follows. The *rhl1⁺* gene was amplified by PCR using the forward primer F-rhl1 [5'-CCCCCGTCGAC(*SalI*)CATGGCTCCTATTAAATC TC-3'] and the reverse primer R-rhl1 [5'-CCCCCGGATCC(*Bam*HI)TTAGGCGATAACACAACCCCTTTCC-3']. The resulting fragment was digested with *Bam*HI and *SalI* and then cloned into pREP41 and pREP81, respectively. pREP41-rhl1^{CVIL} was constructed similarly with the exception of the primer used for PCR that was the reverse primer R-rhl1^{CVIL} [5'-CCCCCGGATCC(*Bam*HI)TTACAAGATAACACAACCC-3'].

Generation of anti-Rhl1 antibody: A His-tagged protein of Rhl1 produced in *Escherichia coli* was used to raise polyclonal antibodies. His-Rhl1 was obtained as follows: A 558-bp DNA fragment carrying the entire *rhl1* coding region was amplified by PCR with two oligonucleotides, 5'-GGGGGATCC(*Bam*HI)GCTCCTATTAAATCTCGTAGAATTG-3' and 5'-CCCCGTCCGAC(*SalI*)TTAGGCGATAACACAACCCCTTTCC-3'. The amplified DNA was digested with *Bam*HI and *SalI* and then inserted into the same sites of the His-tag expression vector pET-30-a to make pET(rhl1). The pET(rhl1) was transformed into *E. coli* Tuner. The fusion protein was purified from the MagneHis Protein Purification System (Promega, Madison, WI) and used to immunize rabbits.

Nucleotide sequence analysis of the *cpp1-1* mutant allele:

The entire *cpp1* ORF was amplified by PCR using genomic DNA prepared from $\Delta tsc2$ *cpp1-1* cells (YKK25) as a template and then cloned into pREP41. The nucleotide sequences of three clones derived from each independent PCR amplification were determined entirely. Comparison of the nucleotide sequences of *cpp1-1* with *cpp1*⁺ revealed a single-nucleotide change, from G to A, which resulted in the replacement of glycine 254 with aspartate.

Western blotting: Total cell lysates were prepared as follows: Cells were lysed with glass beads in lysis buffer [150 mM NaCl and 10 mM Tris-HCl (pH 7.0)] containing 0.5% Triton X-100 and 0.5% deoxycholate. The following protease inhibitors were added to the cell extracts: 0.4 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Nacalai Tesque). Equal amounts of total proteins were then loaded onto a 15% polyacrylamide gel and transferred to nitrocellulose membranes. Antibodies used were anti-Rhb1 and monoclonal TAT-1 for *S. pombe* tubulin (gift from K. Gull, University of Manchester, United Kingdom).

Northern blotting: Total RNAs were prepared from *S. pombe* culture as described (JENSEN *et al.* 1983) and fractionated on a 0.8% gel containing 3.7% formaldehyde gel as previously reported (THOMAS *et al.* 1980). Probes for *fnx1*⁺, *mei2*⁺, and *inv1*⁺ were PCR amplified from a *S. pombe* genomic DNA library and labeled with [α -³²P]dCTP using standard methods.

Spot test: Cells were cultured in liquid YES or EMM medium at a concentration of 1×10^7 cells/ml and each culture was diluted 10, 100, and 1000-fold. Five microliters of each suspension were spotted on appropriate media.

Subcellular fractionation: Spheroplasts were prepared as follows. A total of 10^{10} cells were incubated at 37° for 1 hr in spheroplasts buffer [50 mM citrate-phosphate (pH 5.6) and 1.2 M sorbitol] containing 5 mg/ml lysing enzyme (Sigma, St. Louis). Spheroplasts were resuspended in a lysis buffer [20 mM Hepes-potassium hydroxide (pH 7.5), 20 mM potassium acetate, and 0.1 M sorbitol] containing 0.4 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Nacalai Tesque) and downed ~20 times with a glass tissue homogenizer. The crude lysate was centrifuged at $300 \times g$ to remove unlysed spheroplasts. The $300 \times g$ supernatant was centrifuged at $100,000 \times g$ for 1 hr to separate pellet (P100) and supernatant (S100) fractions.

Microarray analysis: Details of DNA-microarray construction, RNA isolation from fission yeast cells, sample labeling, microarray hybridization, and data processing will be described elsewhere (Y. CHICASHIGE and Y. HIRAOKA, unpublished data). Cells of $\Delta tsc1$ and $\Delta tsc2$ mutants were grown under the reference condition (YES liquid medium) to early-log phase and divided into two parts, one shifted to the experimental condition (3-hr incubation in EMM depleted of nitrogen sources), referred to as mutant/experimental (Mex), and the remainder kept under the reference condition for 3 hr (mutant/reference, Mrf). Poly(A)⁺ RNA was extracted from Mex cells and cDNA probes were prepared, labeled with Cy5, and hybridized to a fission yeast cDNA microarray covering >4900 genes together with Cy3-labeled cDNA probes prepared from Mrf cells. To exclude false positives generated by the experimental condition, we performed the same analysis with a wild-type strain, designating the samples as wild type/experimental (Wex) and wild type/reference (Wrf). After measurement of fluorescent intensity for Cy5 and Cy3, the measured fluorescent intensity, *I*, was corrected as follows to give a corrected intensity, *C*,

$$C = I - M \quad (\text{for } I \geq M + 2s)$$

$$C = I \times 2s / (M + 2s) \quad (\text{for } I \leq M + 2s),$$

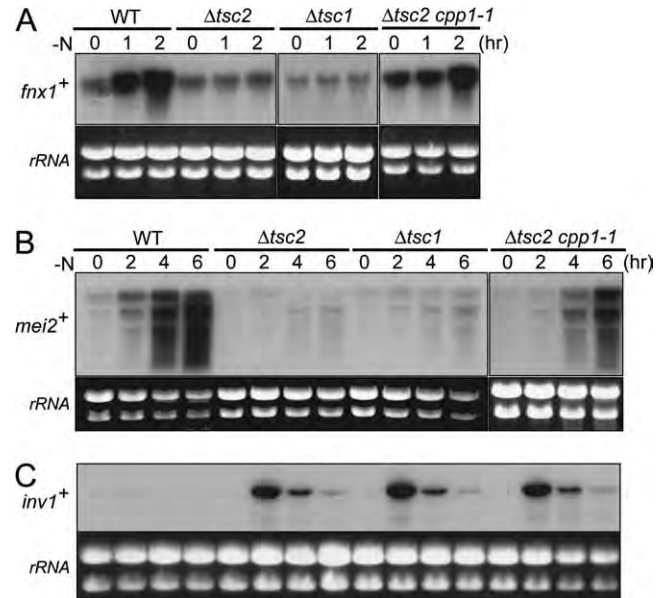


FIGURE 1.—Gene expression upon nitrogen starvation. Wild-type cells (SP6), $\Delta tsc1$ cells (AE413), $\Delta tsc2$ cells (AE512), and $\Delta tsc2$ *cpp1-1* cells (YKK25) transformed with pAL-KS vector were precultured overnight in EMM plus N and then transferred to EMM minus N. Total RNAs were analyzed by Northern blot hybridization with *fnx1*⁺ (A), *mei2*⁺ (B), and *inv1*⁺ (C) as probes.

where *M* and *s* are an average and a standard deviation of *I* of negative control spots for each wave length, respectively. When $I = M + 2s$, that is, $C = 2s$, it was set to be a detection limit. When *C* for either Cy3 or Cy5 or both was $>2s$, the values were considered to be effective data. Expression ratio *r'* of each effective detection spot obtained thus was scaled as follows: $r' = r - m$, $r = \log_2 R$, $R = (C_{cy5} / C_{cy3})$, and *m* is an average of *r* of all effective detection spots.

We identified transcripts that were not induced in each of the two mutants, $\Delta tsc1$ and $\Delta tsc2$, with the following criteria: *r'* of the wild-type strain was >2 whereas that of each mutant was <2 . Transcripts that were more induced in each of the two mutants were identified with the following criteria: *r'* of the mutant strain was >2 whereas that of the wild-type strain was <2 . The original data of microarray experiments have been submitted to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/index.cgi>) and are accessible with accession no. GSE4449.

RESULTS

Gene expression in $\Delta tsc1$ and $\Delta tsc2$: We previously showed that the *sxa2*⁺ gene is not induced in $\Delta tsc1$ (MATSUMOTO *et al.* 2002). Another study (VAN SLEGTHENHORST *et al.* 2004) also indicated that expression of a number of genes in exponentially growing $\Delta tsc1$ and $\Delta tsc2$ cells is abnormal. Having shown that *S. pombe* Tsc1/2 is required for sensing/responding to starvation, we thought that analysis of gene induction/repression upon starvation might provide more insightful information. It was previously demonstrated that expression of *fnx1*⁺ is induced upon nitrogen starvation (DIMITROV and SAZER 1998). As shown in Figure 1,

TABLE 2
Genes not induced in $\Delta tsc1$ and $\Delta tsc2$

ORF ID	Gene name	Possible function
SPCP31B10.09 SPCC962.01		Unknown
SPAC1039.04		Membrane transporter
SPBC1773.17c SPBP26C9.01c		Glycerate and formate dehydrogenase
SPAC1039.01		Amino acid permease
SPBC887.17		Uracil permease
SPAC1399.02		Membrane transporter
SPBP35G2.11c		Zinc finger protein
SPBC947.15c		Mitochondrial NADH dehydrogenase
SPAP7G5.06		Amino acid permease
SPAC1039.03		Esterase/lipase
SPBC24C6.06	<i>gpa1</i>	Guanine nucleotide-binding protein
SPAC13G7.04c	<i>mac1</i>	Membrane-anchored protein
SPAC27F1.05c		Aminotransferase
SPBC1604.03c		Hypothetical protein
SPCC1183.11 SPCC31H12.01		MS ion channel
SPAC31G5.09c	<i>spk1</i>	MAP kinase
SPAC11D3.03c		Conserved protein
SPAC13F5.07c		Hypothetical protein
SPAC27D7.03c	<i>mei2</i>	RNA-binding protein
SPAC11H11.04	<i>mam2</i>	Pheromone P-factor receptor
SPAC186.04c		Pseudogene
SPBC1683.02		Adenosine deaminase
SPBC660.07	<i>ntp1</i>	O-glycosyl hydrolase
SPBC1711.11		Sorting nexin
SPBC36B7.05c		Phosphatidylinositol(3)-phosphate-binding protein
SPBC25B2.02c SPBC2G5.09c	<i>mam1</i>	ABC transporter
SPBPB2B2.01		Amino acid permease
SPCC1682.11c		Hypothetical protein
SPCC550.07		Acetamidase
SPCC550.10	<i>meu8</i>	Betaine aldehyde dehydrogenase
SPCC622.11		Hypothetical protein

ORFs that were induced more than fourfold in the wild type but not in $\Delta tsc1$ or $\Delta tsc2$ 3 hr after nitrogen starvation are listed with their ORF ID, gene name (if available), and possible function.

Northern blot analysis indicated that the level of the induction of *fnx1*⁺ was indeed much lower in $\Delta tsc1$ and $\Delta tsc2$. To obtain genomewide information, we analyzed the expression profile of $\Delta tsc1$ and $\Delta tsc2$ by DNA microarrays. RNAs were prepared from $\Delta tsc1$ and $\Delta tsc2$ cells grown in nitrogen-rich YE medium as well as the cells grown for 3 hr in EMM medium lacking nitrogen. These RNAs were labeled with Cy3 or Cy5 and used as probes for hybridization to DNA microarrays. For the control experiment, we also prepared RNAs from the wild-type strain and used them as probes. Our quantitative analysis indicated that 131 genes are induced upon nitrogen depletion in the wild-type strain, of which ~31 genes are not induced in $\Delta tsc1$ and $\Delta tsc2$ (Table 2). We also found that 32 genes are induced in $\Delta tsc1$ and $\Delta tsc2$, but not in the wild-type strain (Table 3). By performing Northern blots, we confirmed some of the results obtained by the DNA microarray analysis. As shown in Figure 1, *mei2*⁺, which was normally induced upon nitrogen starvation, was not induced in $\Delta tsc1$ and $\Delta tsc2$. Furthermore, *inv1*⁺, which was not expressed at a detect-

able level in the wild-type strain, was induced in $\Delta tsc1$ and $\Delta tsc2$ cells.

Screen for an extragenic suppressor of $\Delta tsc2$: To dissect a genetic pathway involving *tsc1*⁺/*2*⁺, we attempted to isolate extragenic suppressors of $\Delta tsc2$. Assuming that Tsc2 serves as a GAP for Rhb1 GTPase and negatively regulates the Rhb1 function, we were particularly interested in a mechanism to positively regulate the GTPase. If Rhb1 is constitutively active in the $\Delta tsc2$ cells, a loss of function of a gene encoding a positive regulator for Rhb1 would suppress the phenotypes caused by $\Delta tsc2$.

As we previously showed (MATSUMOTO *et al.* 2002), $\Delta tsc2$ *leu1-32* strains are defective in uptake of amino acids and cannot grow on EMM containing leucine at 40 μ g/ml. In the primary screen for the extragenic suppressors of $\Delta tsc2$, we isolated spontaneous revertants, which could grow at 26° on the EMM with leucine at 40 μ g/ml. Sixty-five revertants obtained through the primary screen were tested for their temperature sensitivity at 36° in the secondary screen. Because *rhb1*⁺

TABLE 3
Genes induced more in $\Delta tsc1$ and $\Delta tsc2$ 3 hr after nitrogen starvation

ORF ID	Gene name	Possible function
SPAC21E11.04	<i>ppr1</i>	L-azetidine-2-carboxylic acid acetyltransferase
SPCC1020.14	Tf2-12 tf2-5	tf2-type transposon
SPCC794.05c		Pseudogene
SPAC9.04	Tf2-1 tf2-7	tf2-type transposon
SPAC26A3.13c	Tf2-4 tf2-2	tf2-type transposon
SPCC1494.11c	Tf2-13-pseudo	LTR retrotransposon tf2-type retrotransposon
		polyprotein with 1 frameshift
SPAC167.08 SPAC1F2.03	Tf2-2 tf2-3 tf2-4	tf2-type transposon
SPAC2E1P3.03c SPAC2E1P3.03	tf2-10 Tf2-3	tf2-type transposon
SPBC9B6.02c	tf2-8 Tf2-9	Retrotransposable element
SPAPB18E9.03c		Hypothetical protein
SPBC1E8.04c	Tf2-10-pseudo	Frameshifted LTR retrotransposon polyprotein
SPBC660.09		Hypothetical protein
SPAC3F10.16c		GTPase
SPBC1271.08c		Hypothetical protein
SPBC1271.07c		Acetyltransferase
SPAC57A10.01 SPAC19E9.03	<i>pas1</i>	Pcl-like cyclin
SPBC2G2.04c	<i>mmf1 pmf1</i>	Conserved protein
SPBP4H10.12		Conserved protein
SPAC821.10c	<i>sod1</i>	Cu,Zn-superoxide dismutase
SPBC211.07c	<i>ubc8</i>	Ubiquitin-conjugating enzyme
SPAC29B12.13		Hypothetical protein
SPAC2F3.08	<i>sut1</i>	α -Glucoside transporter
SPCC1450.13c		Riboflavin synthase
SPAC3C7.02c		Hypothetical protein
SPCC70.10		Hypothetical protein
SPAC25B8.09		Methyltransferase
SPCC70.08c		SAM-dependent methyltransferase
SPAC16E8.03	<i>gna1 spgna1</i>	Glucosamine-phosphate N-acetyltransferase
SPBC1773.05c	<i>tms1</i>	Dehydrogenase
SPBC16A3.17c		Transporter
SPBC839.06	<i>cta3</i>	Ca ²⁺ -ATPase
SPCC191.11	<i>inv1</i>	Invertase

ORFs that were induced more than fourfold in $\Delta tsc1$ and $\Delta tsc2$ but not in the wild type 3 hr after nitrogen starvation are listed with ORF ID, gene name (if available), and possible function.

is an essential gene for growth (MACH *et al.* 2000), we postulated that if a revertant carried a mutation on a positive regulator for Rbh1, it might be lethal under a more severe condition. Among the 65 revertants isolated through the primary screen, 11 strains exhibited a temperature sensitivity for growth at 36°. Finally, the revertants, which appeared to be temperature sensitive in the secondary screen, were further tested for their ability to induce *fnx1*⁺ and *mei2*⁺ upon nitrogen starvation. Two revertants satisfied the final criterion and were further examined genetically.

Gene cloning of extragenic suppressor: Genetic analysis indicated that the two revertants carried a single mutation at the same locus responsible for the suppression of $\Delta tsc2$. Furthermore, the suppression activity was found to link to the temperature sensitivity. We attempted to clone the corresponding gene by complementation of the temperature sensitivity and identified six genes. Integration mapping and sequence analysis of

the genome of the suppressors indicated that *cpp1*⁺ is the gene responsible for the suppression. *cpp1*⁺ encodes a β -subunit of a farnesyltransferase (FTase), which farnesylates proteins. The two suppressors of $\Delta tsc2$ carry a mutation at the identical amino acid position Glycine-254 (Figure 2B). We refer to this allele as *cpp1-1* hereafter. Genes encoding the β -subunit of FTase have been identified from yeast to humans. According to a study of the human FTase (PARK *et al.* 1997), the mutation site of *cpp1-1* corresponds to the catalytic center that traps the zinc ion.

The other five genes appeared to be a multicopy suppressor of *cpp1-1*. Overexpression of the *cwp1*⁺ (ARELLANO *et al.* 1998) gene encoding an α -subunit of the FTase suppressed the temperature sensitivity of *cpp1-1* comparatively to that by *cpp1*⁺. The remaining four genes, SPBC36.06c encoding a farnesyl pyrophosphate synthetase, *zfs1*⁺ (KANO *et al.* 1995), *ykt6*⁺ (McNEW *et al.* 1997), and *ste11*⁺ (SUGIMOTO *et al.* 1991), suppressed the

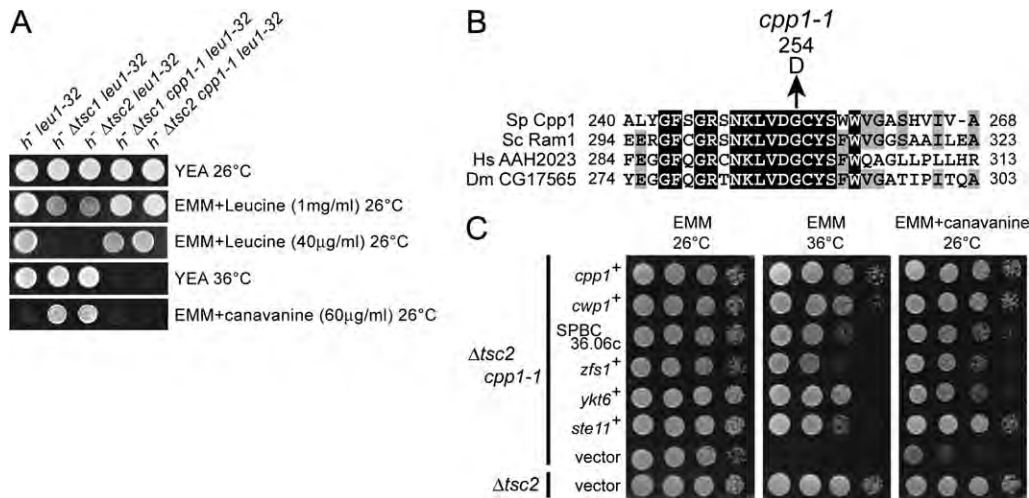


FIGURE 2.—*cpp1-1* is an extragenic suppressor of *Δtsc2*. (A) Genetic interaction among *cpp1-1*, *Δtsc1*, and *Δtsc2*. Each strain was spotted on the indicated media and grown for 2 days (YEA, 36°C) or 3 days (other conditions). (B) Comparison of the amino acid sequences of Cpp1 and other members of β-subunit of FTases. The *cpp1-1* mutant allele carries a single-nucleotide change (from G to A) that results in the replacement of Gly-254 with Asp. Identical amino acids among four species are

shown in white against black and the amino acids conserved among three species are shaded. Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*. (C) The multicopy suppressors of *Δtsc2 cpp1-1* cells. *Δtsc2 cpp1-1* cells carrying each of the five multicopy suppressors were grown in liquid EMM (1×10^7 cells/ml) and spotted on EMM or EMM with canavanine (60 μg/ml). They were incubated for 2 days (EMM, 36°C) or 3 days (EMM or EMM containing canavanine, 26°C).

temperature sensitivity of *cpp1-1* to a lesser extent (Figure 2C and Table 4).

Genetic interaction of *cpp1-1*: The *cpp1-1* allele was originally isolated through a screen for an extragenic suppressor of *Δtsc2*. The two phenotypes associated with *Δtsc2* (i.e., defects in uptake of leucine and gene induction upon nitrogen starvation) were suppressed by this allele. First, *fnx1*⁺ and *mei2*⁺ could be induced upon nitrogen starvation in *Δtsc2 cpp1-1* (Figure 1). Second, *Δtsc2 cpp1-1* double mutants could grow on the EMM containing leucine at 40 μg/ml (Figure 2A). Interestingly, the *cpp1-1* mutation could not suppress the abnormal induction of *inv1*⁺ (Figure 1).

Owing to the defect in uptake, *Δtsc2* cells are resistant to canavanine, a toxic analog of arginine (Figure 2A). The *cpp1-1* mutation abolished the resistance to canavanine in the background of *Δtsc2* (Figure 2A). When the *cpp1*⁺ gene was introduced into a *Δtsc2 cpp1-1* strain, the strain became resistant to canavanine (Figure 2C). Introduction of each of the five multicopy suppressors

into a *Δtsc2 cpp1-1* strain also conferred a resistance to canavanine (Figure 2C).

As it has been postulated that Tsc1 and Tsc2 function together in the same pathway, we were tempted to test if *cpp1-1* could also suppress *Δtsc1*. We found that *Δtsc1 cpp1-1* double mutants could grow on the EMM containing leucine at 40 μg/ml (Figure 2A), indicating that *cpp1-1* can suppress the defect in uptake of *Δtsc1*.

The Cpp1-dependent FTase likely farnesylates a number of proteins (SEBTI 2005). Although suppression of *Δtsc2* by *cpp1-1* suggested that a failure in farnesylation of a protein contributed to the suppression, it was not clear which protein was involved in this process. A likely protein was Rhb1 GTPase, which is believed to be a target of Tsc1/2. Its amino acid sequence ends with the consensus sequence of the FTase substrates, Cys–Val–Ile–Ala (STRICKLAND *et al.* 1998). The previous study (STRICKLAND *et al.* 1998) showed that the requirement of the Cpp1-dependent FTase could be bypassed by alteration of the amino acid sequence of the C terminus

TABLE 4
Multicopy suppressors of *Δtsc2 cpp1-1* cells

Gene name (systematic name)	Accession no.	Possible function
<i>cwp1</i> (SPAPB1A10.04c)	CAC21477.1	α-Subunit of geranylgeranyltransferase I (GGTase I) and farnesyltransferase (FTase)
No name (SPBC36.06c)	CAA19054.1	Farnesyl pyrophosphate (FPP) synthetase
<i>zfs1</i> (SPBC1718.07c)	BAA08654.1	Zinc-finger protein involved in mating and meiosis
<i>ykt6</i> (SPBC13G1.11)	CAA18664.1	Protein with high similarity to <i>S. cerevisiae</i> Ykt6p, which is a synaptobrevin (v-SNARE) homolog that is essential for endoplasmic reticulum–Golgi transport
<i>ste11</i> (SPBC32C12.02)	CAA18162.1	Transcription factor that regulates genes required for mating

Genes responsible for multicopy suppression of *cpp1-1* are listed with their accession number and possible function.

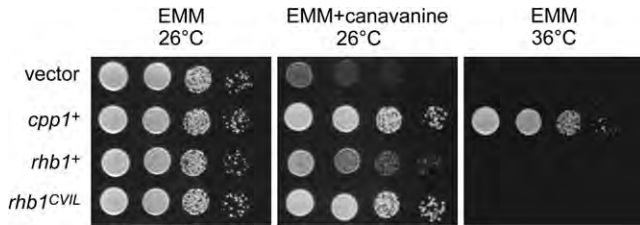


FIGURE 3.—Expression of SpRheb (*rhb1^{CVIL}*). $\Delta tsc2$ *cpp1-1* cells were transformed with vector, plasmids expressing *rhb1+* wild type, *rhb1^{CVIL}* mutant, or *cpp1+* wild type. Transformants were suspended in liquid EMM at a concentration of 1×10^7 cells/ml and 5 μ l of the suspension was spotted on solid EMM or solid EMM containing canavanine (60 μ g/ml). They were incubated for 2 days (EMM, 36°) or 3 days (EMM or EMM containing canavanine, 26°).

to Cys-Val-Ile-Leu, which is recognized by geranylgeranyl transferase, another enzyme to isoprenylate proteins. It was shown previously that proteins, which are normally farnesylated, can remain functional if modified by a geranylgeranyl group (YANG *et al.* 2001). We expressed a mutant of Rhb1-CVIL in a $\Delta tsc2$ *cpp1-1* double mutant, which was sensitive to canavanine, and found that expression of Rhb1-CVIL could confer a resistance to canavanine (Figure 3). The result would suggest that Rhb1 is, at least in part, a protein involved in the suppression of $\Delta tsc2$ by *cpp1-1*. Expression of the mutant Rhb1 did not rescue the temperature sensitivity of the $\Delta tsc2$ *cpp1-1* double mutant, indicating that a failure in farnesylation of another protein caused the temperature sensitivity.

Rhb1 in the *cpp1-1* mutant: Having demonstrated that Rhb1 was involved in the suppression of $\Delta tsc2$, we were prompted to investigate Rhb1 protein biochemically. An antibody to Rhb1 was raised in rabbit and we tested its specificity. As shown in Figure 4, a band corresponding to a 20.5-kDa protein on SDS-PAGE was recognized by the antibody. Because the intensity of this band increased upon overexpression of Rhb1 (Figure 4A), we concluded that the 20.5-kDa protein was Rhb1. To further examine the specificity of the antibody, the antibody was first incubated with an excess amount of recombinant Rhb1 proteins immobilized on beads and the unbound fraction was used for Western blot. The incubation with the Rhb1 beads clearly abolished the 20.5-kDa band, whereas after incubation with beads alone, the antibody could still recognize the 20.5-kDa band (Figure 4B). These results indicated that the antibody specifically recognized the Rhb1 protein in fission yeast cell extracts.

It was likely that the *cpp1-1* mutation causes a defect in the FTase activity and that Rhb1 in the *cpp1-1* background may not be properly modified by a farnesyl group. To test this, we prepared cell extracts from the *cpp1-1* mutant and examined the mobility of Rhb1 on SDS-PAGE. It was shown previously that Rhb1 GTPase

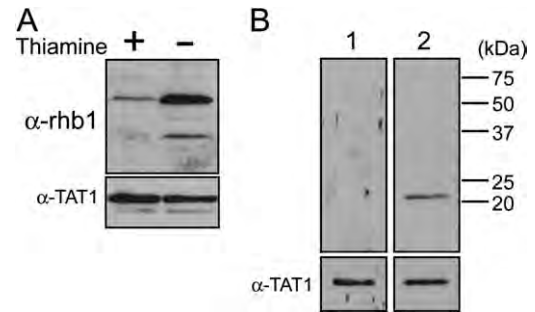


FIGURE 4.—Antibody to Rhb1. (A) Wild-type cells (SP6) carrying pREP81-rhb1 were grown to midlog phase in EMM containing thiamine (50 μ g/ml) and then transferred to thiamine-free EMM for 17 hr at 30°. Protein extracts were subjected to immunoblot analysis with antibody to Rhb1 as well as with anti- α -tubulin antibody (TAT-1) as a loading control. (B) The antibody was first incubated with an excess amount of recombinant Rhb1 proteins immobilized on beads and the unbound fraction was used for Western blot (lane 1). The antibody was incubated with beads alone and the unbound fraction was used for Western blot (lane 2).

in fission yeast (YANG *et al.* 2000) migrates faster on SDS-PAGE if properly modified. As shown in Figure 5A, Rhb1 was detected as a doublet in cell extracts prepared from the *cpp1-1* mutant grown at 26°. Six hours after the shift to the restrictive temperature of 36°, the faster-migrating form of Rhb1 decreased. In cell extracts

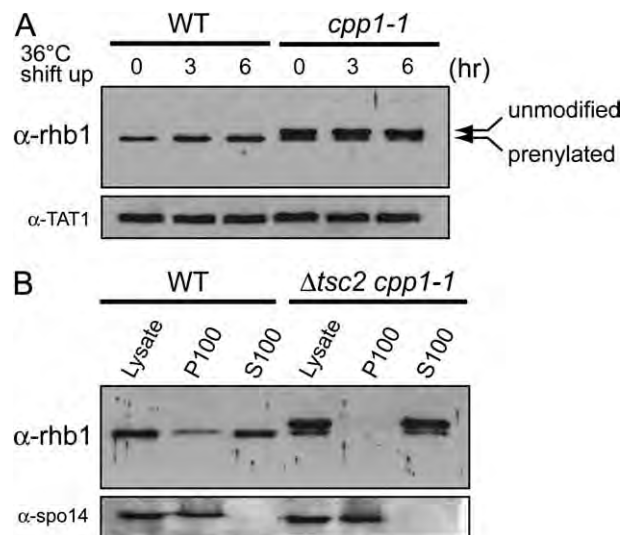


FIGURE 5.—Modification of Rhb1 in *cpp1-1*. (A) Wild-type cells (SP740) and *cpp1-1* cells (YKK55) were grown to midlog phase in YEL medium at 26° and then shifted to 36°. Cell extracts were analyzed with SDS-PAGE and immunoblotted with the anti-Rhb1 and with TAT-1 antibody. (B) Subcellular fractionation of Rhb1. Wild-type cells (SP6) and $\Delta tsc2$ *cpp1-1* cells (YKK25) were grown at 36° for 6 hr. Cells were converted to spheroplasts, homogenized, and subjected to differential centrifugation to fractionate into P100 (membrane fraction) and S100 (supernatant). Each fraction was resolved by SDS-PAGE and subjected to immunoblot analysis using either the anti-Rhb1 or the anti-Spo14 antibody, respectively.

prepared from the wild-type strain, only the faster-migrating form was detected. These results indicated that the *cpl1-1* mutant was indeed defective in protein farnesylation and that the Rhb1 protein was not modified by a farnesyl group.

Farnesylation of the C termini of GTPases is thought to be important for membrane association (CASEY *et al.* 1989). We fractionated cell extracts into a membrane fraction and a cytosolic fraction, and examined which fraction contained Rhb1 GTPase. Spo14, a membrane-bound protein (NAKAMURA-KUBO *et al.* 2003), was used as a probe for the membrane fraction. While a majority of Rhb1 was found in the cytosolic fraction, a small amount of Rhb1 was found in the membrane fraction prepared from the wild-type strain. On the other hand, Rhb1 was not detectable in the membrane fraction prepared from the *cpl1-1* mutant grown at 36° for 6 hr (Figure 5B). These results suggested that only a fraction of Rhb1 is farnesylated and thereby functional in the *cpl1-1* mutant even at the permissive temperature, 26°. We speculate that although the majority of Rhb1 would be a GTP-bound form in $\Delta tsc2$ cells, a failure in farnesylation results in a partial loss of the Rhb1 function, which contributes to the suppression of $\Delta tsc2$.

DISCUSSION

In this study we first analyzed the gene expression profile in $\Delta tsc1$ and $\Delta tsc2$. Second, we demonstrated that a mutation in a gene encoding the β -subunit of FTase can suppress most of the phenotypes associated with a loss of function of Tsc1/Tsc2.

Expression profile of $\Delta tsc1$ and $\Delta tsc2$: The expression profiles of the $\Delta tsc1$ and $\Delta tsc2$ strains examined by the microarrays were very similar each other. We did not find any genes abnormally induced in either one of the two strains. The two genes, *tsc1⁺* and *tsc2⁺*, thereby function together in the same pathway to regulate gene expression upon nitrogen starvation. The genes that cannot be induced in $\Delta tsc1$ and $\Delta tsc2$ are broadly classified in the following groups: genes required for meiosis, genes encoding permeases/transporter for nutrients, and genes encoding enzymes for biosynthesis. The defect in induction of these genes accounts well for the phenotypes of $\Delta tsc1$ and $\Delta tsc2$ (*i.e.*, inefficient meiosis and low uptake).

We also found that 32 genes were induced at higher levels in $\Delta tsc1$ and $\Delta tsc2$ 3 hr after nitrogen starvation. It should be noted that some of these genes may possibly be induced in the wild-type strains at earlier time points and already repressed 3 hr after nitrogen starvation. If their induction is delayed in $\Delta tsc1$ and $\Delta tsc2$ and reaches a peak later, the induction level in $\Delta tsc1$ and $\Delta tsc2$ may be higher than that in the wild-type strain 3 hr after nitrogen starvation. It is, therefore, necessary to examine the induction level of each gene in more detail to identify a gene, which is induced specifically in $\Delta tsc1$

and $\Delta tsc2$. Among the 32 genes that are induced >3 hr after nitrogen starvation in $\Delta tsc1$ and $\Delta tsc2$, we examined *inv1⁺* in detail and demonstrated that it is induced poorly up to 6 hr after nitrogen starvation in the wild-type strain. *inv1⁺* is thereby induced specifically in $\Delta tsc1$ and $\Delta tsc2$. It is normally derepressed upon glucose starvation in fission yeast (TANAKA *et al.* 1998). At present, it is not clear why *inv1⁺* is induced in $\Delta tsc1$ and $\Delta tsc2$ even in the presence of glucose. A factor required for repression of *inv1⁺* may not be expressed in $\Delta tsc1$ and $\Delta tsc2$ when nitrogen is depleted.

Activation of Rhb1 by FTase: Our genetic study demonstrated that a mutation (*cpl1-1*) in the β -subunit of FTase can suppress a loss of function of Tsc1/Tsc2. When a mutant of Rhb1 that bypasses the requirement of farnesylation was expressed, the *cpl1-1* mutation no longer suppressed $\Delta tsc1$ and $\Delta tsc2$. The result indicated that a failure in farnesylation of Rhb1 contributes to the suppression. Only a small fraction of Rhb1 GTPase was found as a modified form in the *cpl1-1* mutant. It has been generally accepted that protein farnesylation at the C terminus of GTPases facilitates membrane association. Consistent with this notion, while a portion of Rhb1 in the wild-type strain was found in the P100 membrane fraction, no Rhb1, in the *cpl1-1* mutant, was found in this fraction. We speculate that activation of Rhb1 requires both GTP binding and farnesylation. The suppression of $\Delta tsc1$ and $\Delta tsc2$ by *cpl1-1* is a result of a decrease in the level of active Rhb1.

Role of the Tsc1/2 complex: Considering that the known biochemical function of the Tsc1/2 complex has so far been to serve as a GAP for the Rhb1 GTPase, it is likely that the defect in gene induction in $\Delta tsc1$ and $\Delta tsc2$ is due to constitutive activation of Rhb1. An active form of Rhb1, in turn, would continuously repress the gene induction even when nitrogen is removed. Supporting this notion, expression of a hyperactive mutant of Rhb1 (URANO *et al.* 2005) resulted in a failure in induction of *mei2⁺* and *fnx1⁺* (K. FUKUDA and T. MATSUMOTO, unpublished results). It has also been reported that the two genes, *mei2⁺* and *fnx1⁺*, are induced upon repression of a hypomorphic allele of *rhb1⁺* in the presence of nitrogen, demonstrating that expression of these genes is solely regulated by Rhb1 (MACH *et al.* 2000). In budding yeast, a number of studies demonstrated that Tor, a downstream target of Rhb1, is involved in transcriptional regulation (BECK and HALL 1999; DUVEL *et al.* 2003; ROHDE and CARDENAS 2003). We speculate that fission yeast Tor1 and Tor2 as targets of Rhb1 play a role in a signal cascade to regulate transcription/translation in response to the availability of nutrients. In this cascade the Tsc1/2 complex regulates Tor1/2 via Rhb1 GTPase.

While the *cpl1-1* mutant clearly restored the ability of $\Delta tsc2$ to induce *mei2⁺* and *fnx1⁺* upon nitrogen starvation, it failed to repress abnormal induction of *inv1⁺* in $\Delta tsc1$ and $\Delta tsc2$. Assuming that the *cpl1-1* mutation can

suppress defects in Rbh1-dependent events, it is possible that the abnormal induction of *inv1*⁺ in $\Delta tsc1$ and $\Delta tsc2$ may not be a result of constitutive activation of Rbh1. In addition to GAP for Rbh1 GTPase, the Tsc1/2 complex may play another role.

TSC pathology and treatment: TSC is a disorder characterized by the wide spread of benign tumors, called hamartomas. The tumor cells exhibit abnormalities in cell size, number, morphology, and location, thereby implying a role of the Tsc1/2 complex in regulating cell growth, proliferation, differentiation, and migration (YEUNG 2003). Because the Tsc1/2 complex regulates protein synthesis via Rheb and mTOR (MANNING and CANTLEY 2003; LI *et al.* 2004a,b; PAN *et al.* 2004), it is currently considered that formation of hamartomas is due to deregulation of protein synthesis.

Our analysis of the expression profile revealed that the $\Delta tsc1$ and $\Delta tsc2$ strains exhibit an abnormality in induction of a number of genes upon nitrogen starvation. They cannot efficiently induce genes required for meiosis, a process of differentiation in fission yeast. We also found that in the $\Delta tsc1$ and $\Delta tsc2$ strains 3 hr after nitrogen starvation, retrotransposons (LTR Tf2) and a G1 cyclin (*pas1*⁺) are expressed at a level higher than that in the wild-type strain. It has been previously reported that *pas1*⁺ is expressed at a higher level in exponentially growing $\Delta tsc1$ and $\Delta tsc2$ strains as well (VAN SLEGTEHORST *et al.* 2005). Although the consequence of abnormal induction of these genes remains to be examined, deregulation of these genes could result in alteration of the genome structure as well as a program of cell proliferation. Expression analysis of the hamartoma cells may allow identification of genes whose abnormal expression accounts for the complex pathology of TSC.

As Rapamycin targets mTOR, it is a good candidate for an anti-TSC drug. On the other hand, it also has immunosuppressive effects (ABRAHAM and WIEDERRECHT 1996), suggesting that it is not an ideal drug for a long term-administration. Our model study in fission yeast demonstrated that a defect in FTase well suppresses the phenotypes associated with deletion of *tsc1/2*⁺. We thereby postulate that an inhibitor of FTase (FTI) should be considered as an anti-TSC drug as well. A combination of Rapamycin and FTI may enhance the specificity of the chemotherapy for TSC. FTIs were originally proposed as anticancer agents because Ras-oncoproteins must be farnesylated for its transforming activity. A number of compounds, some of which competitively inhibit FTase with their structure mimicking the C-terminal C-A-A-X motif of the GTPase, have been developed and tested clinically (OMER and KOHL 1997; GRAAF *et al.* 2004).

The authors are grateful to C. Shimoda and T. Nakamura (Osaka City University, Japan) for providing the antibody to Spo14 and to K. Gull (University of Manchester, United Kingdom) for providing the anti- α -tubulin antibody, TAT-1. The *S. pombe* genomic library was

supplied by National BioResource Project (<http://www.nbrp.jp/>). This work was supported by the U.S. Army Medical Research and Material Command "Tuberous Sclerosis Complex Research Program" (to T.M.) and the Japan Science and Technology Agency (to Y.H.).

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Communicating editor: P. RUSSELL

Distinctive Responses to Nitrogen Starvation in the Dominant Active Mutants of the Fission Yeast Rheb GTPase

Tomoka Murai,* Yukiko Nakase,[†] Keiko Fukuda,*¹ Yuji Chikashige,[‡] Chihiro Tsutsumi,[‡]
Yasushi Hiraoka,^{‡,§} and Tomohiro Matsumoto*^{*,†,2}

*Department of Biostudies, Kyoto University, Kyoto 606-8501, Japan, [†]Radiation Biology Center, Kyoto University, Kyoto 606-8501, Japan,

[‡]Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe 651-2492, Japan and

[§]Graduate School of Frontier Biosciences, Osaka University, Suita 565-0871, Japan

Manuscript received May 22, 2009

Accepted for publication July 16, 2009

ABSTRACT

Rheb, a Ras-like small GTPase conserved from human to yeast, controls Tor kinase and plays a central role in the regulation of cell growth depending on extracellular conditions. Rhb1 (a fission yeast homolog of Rheb) regulates amino acid uptake as well as response to nitrogen starvation. In this study, we generated two mutants, *rhb1-DA4* and *rhb1-DA8*, and characterized them genetically. The V17A mutation within the G1 box defined for the Ras-like GTPases was responsible for *rhb1-DA4* and Q52R I76F within the switch II domain for *rhb1-DA8*. In fission yeast, two events—the induction of the meiosis-initiating gene *mei2⁺* and cell division without cell growth—are a typical response to nitrogen starvation. Under nitrogen-rich conditions, Rheb stimulates Tor kinase, which, in turn, suppresses the response to nitrogen starvation. While amino acid uptake was prevented by both *rhb1-DA4* and *rhb1-DA8* in a dominant fashion, the response to nitrogen starvation was prevented only by *rhb1-DA4*. *rhb1-DA8* thereby allowed genetic dissection of the Rheb-dependent signaling cascade. We postulate that the signaling cascade may branch below Rhb1 or Tor2 and regulate the amino acid uptake and response to nitrogen starvation independently.

SIGNALING involving mTOR kinase is evolutionally conserved from yeast to human. In vertebrates, it regulates cell growth depending on the availability of nutrients, energy source, and growth factors (HAY and SONENBERG 2004; WULLSCHLEGER *et al.* 2006; GUERTIN and SABATINI 2007). The mTOR signaling involves a Ras-like GTPase, Rheb that promotes protein synthesis and thereby cell growth via mTOR (SAUCEDO *et al.* 2003; STOCKER *et al.* 2003). When the environment surrounding the cell is not favorable for growth and proliferation, the protein complex of TSC1 and TSC2, which has a GAP GTPase-activating protein (GAP) activity, converts Rheb into a GDP-bound form (INOKI *et al.* 2003; TEE *et al.* 2003; ZHANG *et al.* 2003; LI *et al.* 2004b). A loss of TSC1/2 would thereby result in constitutive upregulation of the Rheb GTPase and of mTOR and, in humans, allow the wide spread of benign tumors termed “hamartomas” in different organs including the brain, eyes, heart, kidney, skin, and lungs (MANNING and CANTLEY 2003; LI *et al.* 2004a; PAN *et al.* 2004).

In fission yeast, a prototype of the TOR-signaling system consists of Tsc1/2, Rhb1 GTPase (a homolog of Rheb GTPase), and Tor2 kinase (a homolog of mTOR),

as in the mTOR-signaling system in higher eukaryotes (OTSUBO and YAMAMATO 2008). It has been demonstrated by a number of studies that the Tor signaling in fission yeast controls various biological responses to starvation of nitrogen. Upon starvation of nitrogen, fission yeast cells are arrested at G1 with small- and round-cell morphology following two rounds of rapid cell cycling without net growth (YOUNG and FANTES 1987). They also induce expression of the *mei2⁺* gene that is required for initiation of meiosis (WATANABE *et al.* 1988). Upon a loss of function of *tor2⁺* gene, these events are induced even under a nitrogen-rich condition, indicating that Tor2 is responsible for suppression of the events that are normally induced upon nitrogen starvation (ALVAREZ and MORENO 2006; URITANI *et al.* 2006; HAYASHI *et al.* 2007; WEISMAN *et al.* 2007; MATSUO *et al.* 2007). The events adaptive to nitrogen starvation are important for the preservation and survival of the organism, but they are deleterious for actively growing cells. Thus, it is critical for the cell to strictly control the activity of the Tor2 kinase depending on the nutrient condition.

Rheb/Rhb1 GTPase plays a central role as a signal carrier in the Tor signaling. In *Drosophila*, it has been shown that Rheb promotes cell growth via the Tor-signaling system (SAUCEDO *et al.* 2003; STOCKER *et al.* 2003). In fission yeast, a loss of *rhb1⁺* causes a cell cycle arrest at G1 and induction of *fnx1⁺* and *mei2⁺* genes, both of which are normally suppressed by Tor2 (MACH

¹Present address: Yamada Bee Farm, 194 Ichiba, Kagamino-cho Tomadagun, Okayama 708-0393, Japan.

²Corresponding author: Yoshida-Konoe cho, Sakyo Ku, Kyoto 606-8501, Japan. E-mail: tmatsumo@house.rbc.kyoto-u.ac.jp

et al. 2000; MATSUO *et al.* 2007). These genetic studies have indicated that Rheb/Rhb1 positively regulates Tor kinase. Biochemical studies have also demonstrated close interaction between Rheb/Rhb1 GTPase and Tor kinase. It has been shown that when Rheb is ectopically expressed, it binds to mTOR (LONG *et al.* 2005). Subsequently, it has been demonstrated that purified Rheb loaded with GTP, but not with GDP, activates mTOR kinase *in vitro* (SANCAK *et al.* 2007). Ras-like small GTPases, in general, bind to and stimulate their targets only when they are loaded with GTP. The above two studies, however, have reported that binding between Rheb and mTOR is not influenced by the guanine-nucleotide binding state of Rheb. In contrast, Rhb1 in a GTP-bound form binds to Tor2 kinase in fission yeast (URANO *et al.* 2005; URITANI *et al.* 2006). Another study has demonstrated that GTP-bound Rheb directly binds to FKBP38, a member of the FK506-binding protein, and protects mTOR from inhibition by FKBP38 (BAI *et al.* 2007), suggesting that Rheb activates mTOR rather indirectly, although this model has been disputed by a recent study (WANG *et al.* 2008). Obviously, further investigation is needed to formulate a general view of a molecular mechanism by which Rheb/Rhb1 GTPase interacts with Tor as well as with other components.

In our previous studies, we showed that deletion of the *tsc2⁺* gene encoding a GAP for Rhb1 GTPase confers a defect in uptake of amino acids (MATSUMOTO *et al.* 2002) and prevents induction of the *mei2⁺* gene upon nitrogen starvation (NAKASE *et al.* 2006). Rhb1 GTPase, which is assumed to be in a GTP-bound form in cells lacking Tsc2 ($\Delta tsc2$), is likely a cause of these defects. In this study, we generated two dominant active *rhb1* mutants and found that they distinctively respond to nitrogen starvation. One mutant (*rhb1-DA4*) exhibits the phenotypes identical to that of $\Delta tsc2$. The other one (*rhb1-DA8*), however, is defective in uptake of amino acids, but allows induction of the *mei2⁺* gene upon nitrogen starvation. Remarkably, *rhb1-DA8* also allows induction of the *mei2⁺* gene in the $\Delta tsc2$ background. On the basis of these results, we propose a model for Rhb1 signaling in fission yeast.

MATERIALS AND METHODS

Yeast strains, media, and transformation: The *Schizosaccharomyces pombe* strains used in this study are listed in Table 1. The yeast cells were grown in yeast extract with supplements (YES) media and EMM synthetic minimal media with appropriate nutrient supplements as described by MORENO *et al.* (1991). All yeast transformations were carried out by lithium acetate methods (OKAZAKI *et al.* 1990; GIETZ *et al.* 1992).

Mutagenesis of *rhb1⁺*: The *rhb1⁺* gene was amplified by PCR using the forward primer 5'-*rhb1* [5'-GGGGGGGGTTCGAC (*SalI*)ATGGCTCCTATTAAATCTCGTAGA-3'] and the reverse primer 3'-*rhb1* [5'-CCCCCCCCGGATCC (*Bam*HI)TTAGGCGATAACACACCCCTTTCC-3']. To introduce mutations, PCR was performed in the presence of 0.5 mM Mn²⁺ and 1 mM

TABLE 1
Strains used in this study

Strain	Genotype	Source and reference
972h ⁻	<i>h⁻</i>	Laboratory stock
AE411	<i>h⁻ tsc1::ura4⁺ ura4-D18</i>	Laboratory stock
AE509	<i>h⁻ tsc2::ura4⁺ ura4-D18</i>	Laboratory stock
AE512	<i>h⁻ tsc2::ura4⁺ leu1-32 ura4-D18</i>	Laboratory stock
JT177	<i>h⁹⁰ tor2-ts6 ade6-M216 leu1-32 ura4-D18</i>	MATSUO <i>et al.</i> (2007)
JT178	<i>h⁹⁰ tor2-ts10 ade6-M210 leu1-32 ura4-D18</i>	MATSUO <i>et al.</i> (2007)
JW952	<i>h⁻ tor1::ura4⁺ ade6-M216 leu1-32 ura4-D18</i>	MATSUO <i>et al.</i> (2003)
M5-1e1	<i>h⁻ rhb1-DA4 leu1-32</i>	This work
M5-2g3	<i>h⁻ rhb1-DA4</i>	This work
M6-1	<i>h⁺/h⁻ ade6-M210/ade6-M216</i>	This work
M6-13	<i>h⁺/h⁻ rhb1⁺/rhb1-DA4 ade6-M210/ade6-M216</i>	This work
M7-1	<i>h⁻ rhb1-DA4 cpp1-1</i>	This work
M20-76	<i>h⁻ rhb1-DA8 leu1-32</i>	This work
M21-13	<i>h⁻ rhb1-DA8</i>	This work
M38-9	<i>h⁻ rhb1-DA8 cpp1-1</i>	This work
M27-1	<i>h⁺/h⁻ rhb1⁺/rhb1-DA8 ade6-M210/ade6-M216</i>	This work
M47-25	<i>h⁻ tsc2::ura4⁺ rhb1-DA8 ura4-D18</i>	This work
M86-20	<i>h⁻ tor2-ts6 rhb1-DA4</i>	This work
M87-18	<i>tor1::ura4⁺ ura4-D18^a</i>	This work
M87-7	<i>tor1::ura4⁺ rhb1-DA4 ura4-D18^a</i>	This work
M88-23	<i>tor1::ura4⁺ rhb1-DA8 ura4-D18^a</i>	This work
SP6	<i>h⁻ leu1-32</i>	Laboratory stock
YKK90	<i>h⁻ cpp1-1</i>	Laboratory stock
YKK273	<i>h⁻ tor2-ts6 leu1-32</i>	Laboratory stock
YKK276	<i>h⁺ tor2-ts10</i>	Laboratory stock
YKK278	<i>h⁺ tsc2::ura4⁺ tor2-ts6 ura4-D18</i>	Laboratory stock
YKK282	<i>h⁺ tor2-ts6</i>	Laboratory stock
YKK418	<i>tor1::ura4⁺ tsc2::ura4⁺ ura4-D18^a</i>	Laboratory stock

^a Mating type was not determined.

Mg²⁺. The resulting fragments were digested with *Bam*HI and *Sal*I and then cloned into pREP81 to construct a pool of *rhb1* mutants.

Deletion of *rhb1⁺* gene: A plasmid to delete the *rhb1⁺* gene, pBS- $\Delta rhb1$, was constructed by PCR amplification of a 1.1-kbp DNA fragment upstream of the start codon of the *rhb1⁺* gene and a 1.4-kbp DNA fragment downstream of the stop codon. The primer sequences used in the PCR were as follows: for amplification of the upstream sequence, the forward primer was *rhb1-NotI-1* [5'-CCCCCGCGGCCGC (*NotI*)GTG TAAAGGGAGCCGTTCAAG-3'] and the reverse primer was *rhb1-BamHI-2* [5'-CCCCCGGATCC (*Bam*HI)GGCAAAT TATTAAGTATAGAG-3']; for the downstream sequence, the forward primer was *rhb1-BamHI-3* [5'-CCCCCGGATCC (*Bam*HI)GCTTCTGCTTGAATTATC-3'] and the reverse primer was *rhb1-SalI-4* [5'-CCCCCGTCCGAC (*Sal*I)GTACGTT

CAATTCCTATTC-3']. The resulting DNA fragments were digested with combinations of appropriate restriction enzymes and then ligated into pBS plasmids to create pBS-*rhb1*-up and pBS-*rhb1*-down. The pBS-*rhb1*-down plasmid was digested with *NotI* and *Bam*HI and ligated with a *NotI*-*Bam*HI fragment that contained the *rhb1* upstream sequence isolated from pBS-*rhb1*-up to create pBS-*rhb1*-up-down. pBS-*rhb1*-up-down was digested with *Bam*HI and ligated with a DNA fragment that contained the *ura4⁺* gene to create pBS- Δ *rhb1*. Digestion of pBS- Δ *rhb1* with *NotI* and *SalI* generated a 4.3-kbp DNA fragment containing the *ura4⁺* gene flanked by the 1.1-kbp upstream and the 1.4-kbp downstream sequences of the *rhb1⁺* gene, which was used for transformation to delete the *rhb1⁺* gene in a diploid strain.

Spot test: Cells were cultured in liquid YES or EMM medium at a concentration of 1×10^7 cells/ml, and each culture was diluted by a factor of 10. Five microliters of each suspension was spotted onto appropriate media.

Western blotting: Total cell lysates were prepared as follows. Cells were lysed with glass beads in $1 \times$ PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , and 8.1 mM Na_2HPO_4) containing 1 mM MgCl_2 , 0.5% Triton X-100, and 0.5% deoxycholate. The following protease inhibitors were added to the cell extracts: 1 mM phenylmethylsulfonyl fluoride and $1 \times$ protease inhibitor cocktail (Nacalai Tesque). Equal amounts of total proteins were then loaded onto a 15% polyacrylamide gel and transferred to nitrocellulose membranes. Filters were probed with anti-Rhb1 rabbit polyclonal antibodies (NAKASE *et al.* 2006) at a 1:2000 dilution. Blots were also probed with an anti- α -tubulin monoclonal antibody, TAT-1 (a gift from K. Gull, University of Manchester, Manchester, UK), to normalize protein loading. Other procedures used were Northern blotting and microarray analysis, which were performed as described previously (NAKASE *et al.* 2006; CHIKASHIGE *et al.* 2007).

RESULTS

Screen for *rhb1* mutants: We and other groups previously showed that a loss of Tsc2 (GTPase-activating protein for Rhb1 GTPase) causes a reduction in the uptake of amino acids and resistance to canavanine, a toxic analog of arginine (VAN SLEGTHENHORST *et al.* 2004; NAKASE *et al.* 2006). Rheb GTPase, which would predominantly remain as a GTP-bound form in the cells lacking the Tsc2 activity, is likely a cause of the low uptake of amino acids and the resistance to canavanine. We reasoned that a mutation on the *rhb1⁺* gene, which can stably maintain bound GTP or abnormally activate a downstream component, might confer a phenotype similar to that observed in cells lacking Tsc2 (Δ *tsc2*). To obtain such a mutation, a pool of the *rhb1* mutants was generated by error-prone PCR. Individual PCR products were cloned into a plasmid, pREP81, which allowed expression of a cloned gene from an inducible promoter, *nmt1*, and transformed into a wild-type strain. We screened for transformants, which became resistant to canavanine upon expression of the cloned *rhb1* mutant. Among $\sim 40,000$ transformants, we found 3 exhibiting strong resistance to canavanine upon expression of the *rhb1* gene, which presumably carried a mutation.

Construction of *rhb1*-DA4 and *rhb1*-DA8: The plasmids were recovered from the three transformants, and

the nucleotide sequence of the *rhb1⁺* gene (designated *rhb1*-DA4, -DA5, and -DA8, respectively) was analyzed. Because we found that the *rhb1* gene isolated from each transformant carried multiple mutations, we determined the mutation sites responsible for conferring the resistance to canavanine. As shown in Figure 1A, the mutation of *rhb1*-DA4 responsible for conferring the resistance to canavanine is valine at position 17 replaced with alanine (V17A). The *rhb1*-DA5 carries the responsible mutation at the same position, but replaced with aspartic acid (V17D). The *rhb1*-DA8 carries two mutations replacing glutamine at position 52 with arginine (Q52R) and isoleucine at position 76 with phenylalanine (I76F), both of which are necessary for conferring the resistance to canavanine (Figure 1A).

A previous report (URANO *et al.* 2005) described several hyperactive mutations of the fission yeast *rhb1* gene, including V17A (identical to *rhb1*-DA4) and V17G within the G1 box, a domain important for guanine nucleotide binding (Figure 1B and see DISCUSSION). Biochemical analysis indicated that the V17G mutation inhibits both GTP and GDP binding. It is thereby plausible that Rhb1 GTPase stimulates its effector regardless of the guanine-nucleotide-binding status if valine at position 17 is mutated to glycine or alanine. The *rhb1*-DA8 contains mutations at Q52 and I76, both of which are not conserved in other small GTPases and thus its biochemical character is less predictable.

We introduced the *rhb1*-DA4 and *rhb1*-DA8 to the native *rhb1* locus. In the heterozygous diploid strain *rhb1⁺/Δrhb1*, the deletion allele (Δ *rhb1*) was replaced with either the *rhb1*-DA4 allele or the *rhb1*-DA8 allele. The resulting heterozygous diploids were sporulated, and each of them was found to successfully produce four viable spores. We determined the genotype of each cell by sequencing the *rhb1* locus and confirmed that each diploid produced two wild-type cells and two *rhb1* mutants.

Genetic analysis of *rhb1*-DA4 and *rhb1*-DA8: We examined the *rhb1*-DA4 and *rhb1*-DA8 mutants for their resistance to canavanine. As shown in Figure 2A, the two mutants were resistant to canavanine, although the *rhb1*-DA4 mutant was resistant to a lesser extent. We also found that these mutants were defective in uptake of leucine (Figure 2B). The heterozygous diploids *rhb1⁺/rhb1*-DA4 and *rhb1⁺/rhb1*-DA8 were also resistant to canavanine (Figure 2C), indicating that these two mutations are dominant.

We previously showed that a loss of the *cpl1⁺* gene (*cpl1*-1) suppresses defects associated with Δ *tsc2* (NAKASE *et al.* 2006). The *cpl1⁺* gene encodes an enzyme that farnesylates the C terminus of Rhb1 GTPase. We speculate that a failure in farnesylation results in a partial loss of the Rhb1 function, which contributes to the suppression of Δ *tsc2*. To test whether the *cpl1*-1 mutation could suppress *rhb1*-DA4 and *rhb1*-DA8, it was introduced into each of the *rhb1* mutants. As shown in

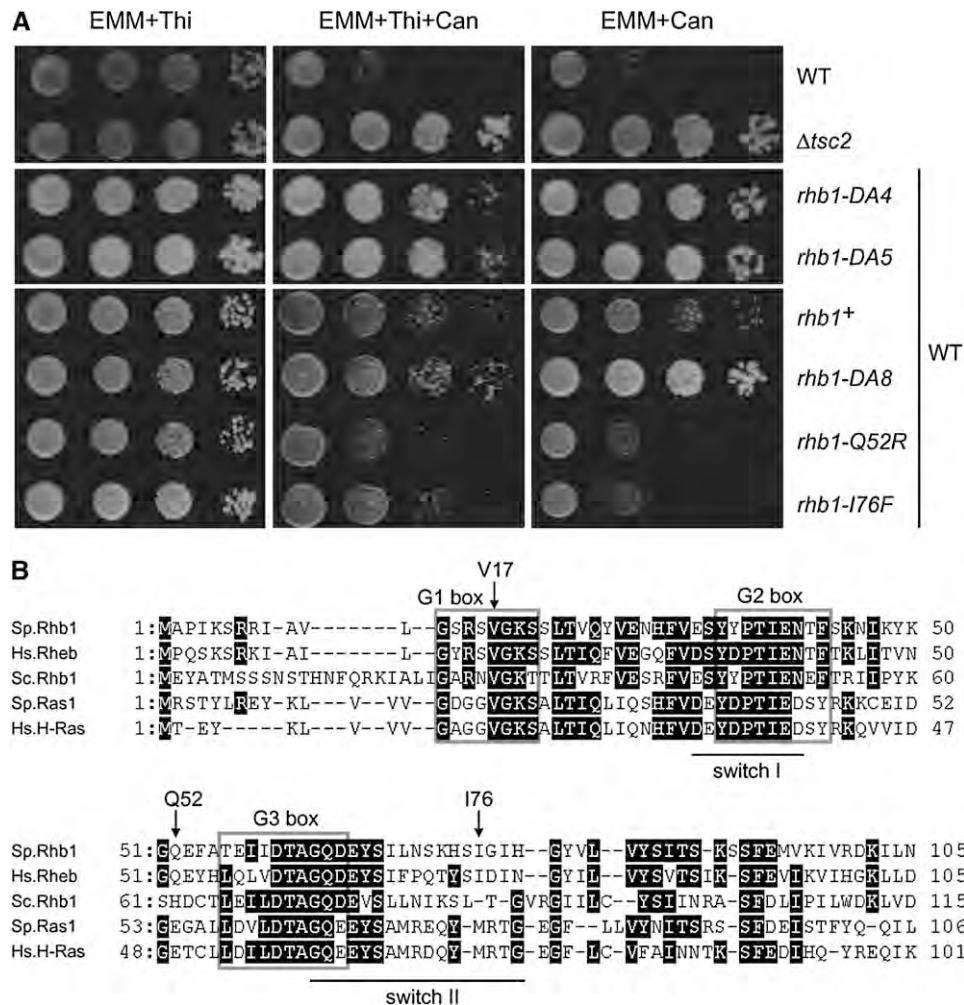


FIGURE 1.—Isolation of *rhb1-DA4* and *rhb1-DA8*. (A) A wild-type strain (972h⁺), a strain deleted for *tsc2*⁺, $\Delta tsc2$ (AE509), and a wild-type strain (SP6) carrying pREP81-*rhb1*⁺, pREP81-*rhb1-DA4*, pREP81-*rhb1-DA5*, pREP81-*rhb1-DA8*, pREP81-*rhb1-Q52R*, or pREP81-*rhb1-I76F* were examined for their resistance to canavanine (Can) at a concentration of 60 μ g/ml by spot test. They were grown in liquid EMM (1 $\times$ 10⁷ cells/ml) and spotted on EMM media without or with thiamine (Thi) at a concentration of 50 μ g/ml, which represses expression of the cloned *rhb1*⁺ gene from the *nmt1* promoter. The plates were incubated at 30° for 3 days without canavanine and 5 days with canavanine. The *rhb1-Q52R* and *rhb1-I76F* mutants each contained one of the two mutations found in the *rhb1-DA8* mutation. (B) A partial amino acid sequence of fission yeast Rhb1 is aligned with those of other GTPases, human Rheb (Hs.Rheb), budding yeast Rhb1 (Sc.Rhb1), fission yeast Ras1 (Sp.Ras1), and human H-Ras (Hs.H-Ras). V17 was substituted with alanine in the *rhb1-DA4* mutant, and Q52 and I76 with arginine and phenylalanine, respectively, in the *rhb1-DA8* mutant.

Figure 2D, the *cpl1-1* mutation fully suppressed the resistance to canavanine in *rhb1-DA4*. In contrast, the suppression of the resistance by the *cpl1-1* mutation was only partial in the *rhb1-DA8* mutant. We also examined Rhb1-DA4 and Rhb1-DA8 for their modification by Cpp1. It was shown previously that Rhb1 GTPase in fission yeast (YANG *et al.* 2000) migrates faster on SDS-PAGE if properly modified. As shown in Figure 2E, Rhb1, Rhb1-DA4, and Rhb1-DA8 were detected as a doublet in cell extracts prepared from the *cpl1-1* mutant grown at 26°. Three hours after the shift to the restrictive temperature 36°, the slower-migrating form of Rhb1 increased. In cell extracts prepared from the wild-type background, only faster-migrating forms of Rhb1-DA4 and Rhb1-DA8 were detected. These results indicated that Rhb1-DA8 was not fully modified with a farnesyl group in the *cpl1-1* mutant even at 26°. It is thus likely that Rhb1-DA8 GTPase is less dependent on the modification by Cpp1 to cause the resistance to canavanine.

Response to nitrogen starvation: Fission yeast cells, when grown in nitrogen-free media, undergo stimulated rates of division mostly due to a shortened G2 phase, a period important for cell growth for this organism (YOUNG and FANTES 1987). As a result, cells

become small and round. They also allow induction of the meiosis-initiating gene *mei2*⁺. These two events are a hallmark of the response to nitrogen starvation in fission yeast. In this study, the two dominant active *rhb1* mutants, along with $\Delta tsc2$, were examined for their response to nitrogen starvation in detail.

We first examined cell morphology and rates of cell division after nitrogen starvation. As shown in Figure 3, 4 hr after the shift to the nitrogen-free media, the wild-type cells became round and small. Its growth rate was stimulated upon nitrogen starvation (Figure 4). In contrast, $\Delta tsc2$ and the *rhb1-DA4* mutant responded minimally to nitrogen starvation. Their morphology did not change dramatically (Figure 3), and their rates of division were lower in the absence of a nitrogen source (Figure 4). We also examined the *rhb1-DA8* mutant and unexpectedly found that it behaved just like the wild-type strain in the nitrogen-free media. As shown in Figures 3 and 4, it exhibited shorter and round morphology after stimulated cell division upon the shift to the nitrogen-free media.

We next examined expression of three genes, which are abnormally expressed in $\Delta tsc2$ upon the shift to the nitrogen-free media. The *mei2*⁺ gene that is induced in

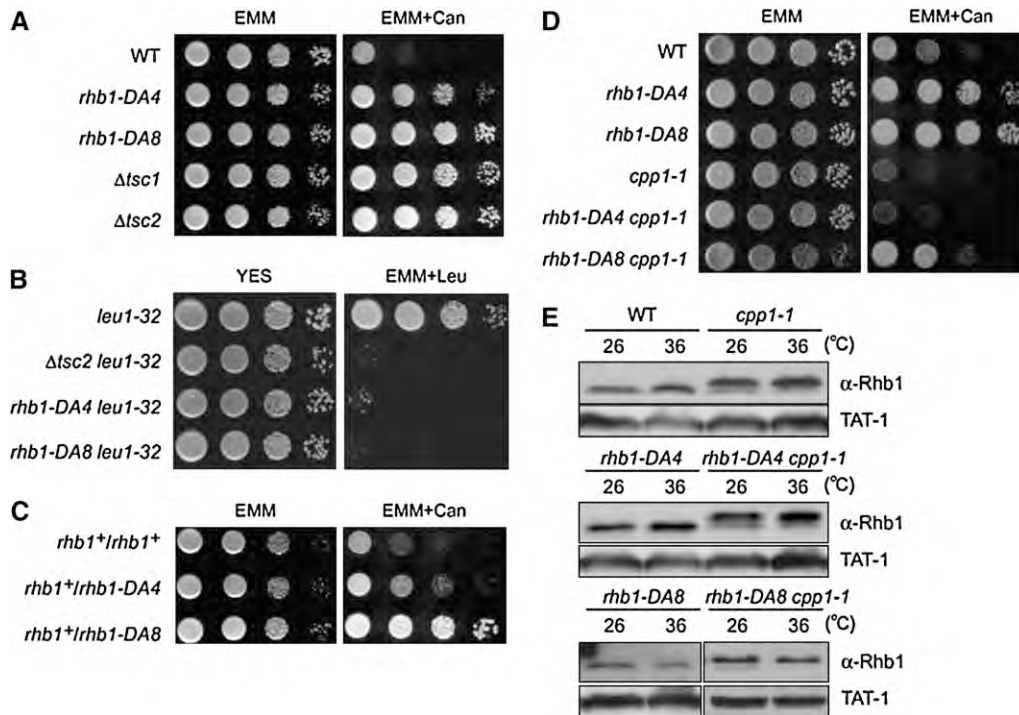


FIGURE 2.—Defect in amino acid uptake and resistance to canavanine. (A) Wild-type cells (972h⁻), *rhb1-DA4* cells (M5-2g3), *rhb1-DA8* cells (M21-13), $\Delta tsc1$ cells (AE411), and $\Delta tsc2$ cells (AE509) were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test. They were grown in liquid EMM (1×10^7 cells/ml) and spotted on EMM media without or with canavanine at a concentration of 60 μ g/ml. The plates were incubated at 30° for 3 days without canavanine and 5 days with canavanine. (B) Wild-type cells (SP6), $\Delta tsc2$ cells (AE512), *rhb1-DA4* cells (M5-1e1), and *rhb1-DA8* cells (M20-76) were examined for the ability to take up leucine by spot test. They were grown in liquid

YES (1×10^7 cells/ml) and spotted on YES or EMM media containing leucine at a concentration of 40 μ g/ml (EMM + Leu). The plates were incubated at 30° for 2 days (YES) or 3 days (EMM + Leu). (C) Wild-type diploid cells (M6-1), *rhb1*⁺/*rhb1-DA4* heterozygous diploid cells (M6-13), and *rhb1*⁺/*rhb1-DA8* heterozygous diploid cells (M27-1) were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test as in A. (D) Wild-type cells (972h⁻), *rhb1-DA4* cells (M5-2g3), *rhb1-DA8* cells (M21-13), *cpp1-1* cells (YKK90), *rhb1-DA4 cpp1-1* cells (M7-1), and *rhb1-DA8 cpp1-1* cells (M38-9) were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test as in A. The plates were incubated at 26° for 3 days without canavanine and 5 days with canavanine. (E) Wild-type cells (972h⁻), *rhb1-DA4* cells (M5-2g3), *rhb1-DA8* cells (M21-13), *cpp1-1* cells (YKK90), *rhb1-DA4 cpp1-1* cells (M7-1), and *rhb1-DA8 cpp1-1* cells (M38-9) were grown to midlog phase in YES media at 26° and then shifted to 36°. They were incubated for 3 hr at 36°. Cell extracts were analyzed with SDS-PAGE and immunoblotted with antibodies to Rhb1. The level of α -tubulin (loading control) was monitored with the antibody, TAT-1.

the wild-type strain is not induced in $\Delta tsc2$. On the other hand, the *inv1*⁺ gene and Tf2 (a transposable element) that are not induced in the wild-type strain are induced in $\Delta tsc2$ (NAKASE *et al.* 2006).

As shown in Figure 5A, in the *rhb1-DA4* mutant, the *mei2*⁺ gene was not induced and the *inv1*⁺ gene and Tf2 were induced. In addition, we found that introduction of *cpp1-1*, a suppressor of $\Delta tsc2$, also suppressed the phenotype of *rhb1-DA4* (Figure 5B). These results indicated that the *rhb1-DA4* mutation causes phenotypes indistinguishable from that of $\Delta tsc2$. In contrast, expression of the three genes was normal in *rhb1-DA8*. Upon nitrogen starvation, the *mei2*⁺ gene was induced and the two genes, *inv1*⁺ and Tf2, were not (Figure 5A). We also analyzed the gene expression profile in a genomewide scale by microarrays. As shown in Table 2, while a number of genes were abnormally expressed in $\Delta tsc2$ and the *rhb1-DA4* mutant, they were normally expressed in the *rhb1-DA8* mutant.

Suppression of $\Delta tsc2$ by *rhb1-DA8*: The results described above uncovered a very unique feature of the *rhb1-DA8* mutation. Although it strongly confers the resistance to canavanine in a dominant manner, the *rhb1-DA8* mutation does not affect the responses to

nitrogen starvation that are induction of the *mei2*⁺ gene and stimulated cell division with no net growth. Because these two events are regulated by the Tor2 kinase (ALVAREZ and MORENO 2006; URITANI *et al.* 2006; HAYASHI *et al.* 2007; MATSUO *et al.* 2007; WEISMAN *et al.* 2007), we speculated that (1) the *rhb1*-signaling cascade branches into two pathways, the Tor2-dependent pathway and an unknown one, and (2) the Rhb1-DA8 GTPase might preferentially stimulate the unknown target that is responsible for conferring the resistance to canavanine. To further understand the nature of the *rhb1-DA8* mutation, we constructed a double mutant, $\Delta tsc2$ *rhb1-DA8*, and examined it for the response to nitrogen starvation.

As shown in Figure 4 and Figure 6A, upon the shift to the nitrogen-free media, the double mutant became shorter and round following a stimulated division. It also allowed induction of the *mei2*⁺ gene (Figure 6B). These results suggested that the Rhb1 GTPase, which would remain in a GTP-bound form in $\Delta tsc2$, cannot stimulate the Tor2 kinase in the *rhb1-DA8* background.

Interaction between Tor and Rhb1-DA: To further explore the functional relationship between Tor2 and Rhb1, we attempted to examine the effect of the *rhb1*-

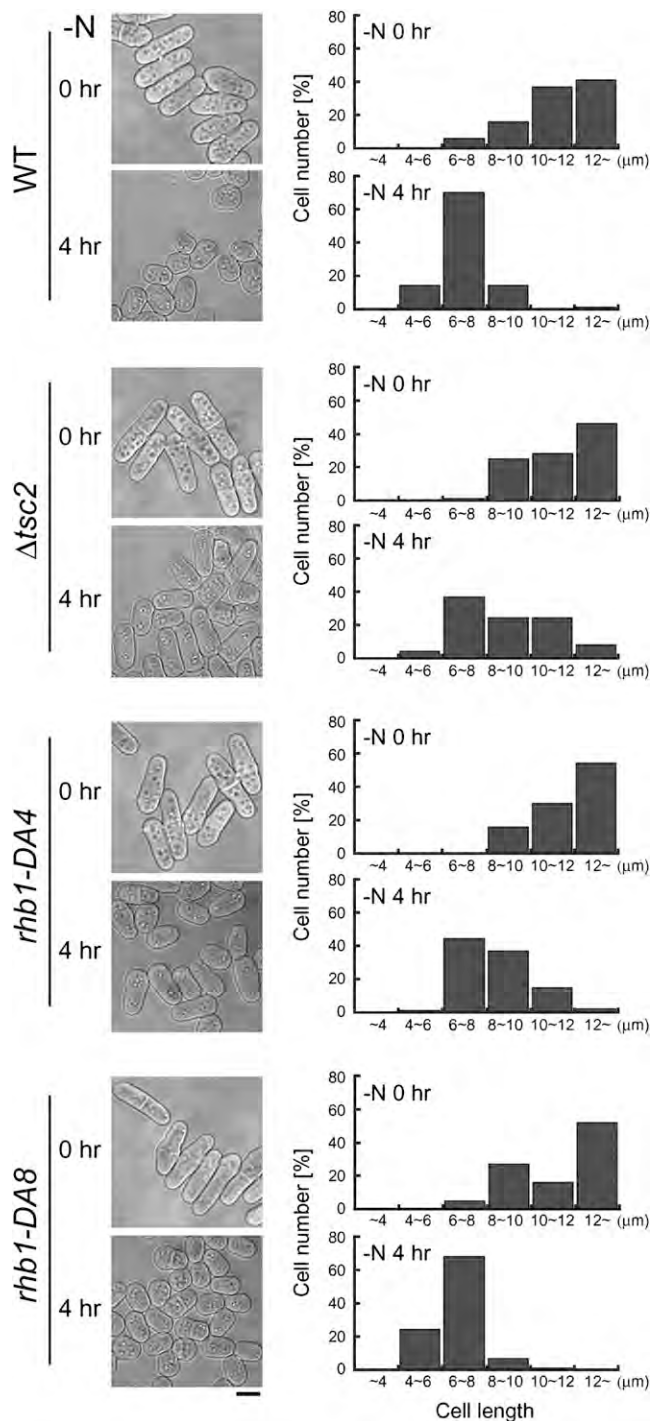


FIGURE 3.—Cell division after nitrogen starvation. Wild-type cells (972h⁻), $\Delta tsc2$ cells (AE509), *rhb1-DA4* cells (M5-2g3), and *rhb1-DA8* cells (M21-13) were grown in the presence of a nitrogen source, 93.5 mM NH₄Cl (0 hr), and shifted to the media lacking a nitrogen source at 26° for 4 hr. Their morphology was observed under a microscope (left panels), and the distribution of their cell length was measured (right panels). For each strain, >100 cells were counted. The experiments were performed three times with reproducible results. The results presented are from a representative single experiment. Bar, 5 μm.

DA4 and *rhb1-DA8* mutations on a temperature-sensitive allele of *tor2*, *tor2-ts6*, or *tor2-ts10* (MATSUO *et al.* 2007). As shown in Figure 7A, the *tor2-ts6* mutant was resistant to canavanine, but to a lesser extent compared with the *rhb1-DA4* or $\Delta tsc2$ single mutants. We introduced *rhb1-DA4*, a mutation previously characterized as hyperactive (URANO *et al.* 2005), into *tor2-ts6* and found that the double mutant exhibited a canavanine resistance similar to that of the *rhb1-DA4* single mutant (Figure 7A). We also constructed the double mutant *tor2-ts6* $\Delta tsc2$ and found that it exhibited a resistance to canavanine similar to that of the $\Delta tsc2$ mutant. These results thereby indicated that the resistance to the drug conferred by *rhb1-DA4* or $\Delta tsc2$ was not influenced by *tor2-ts6*. The resistance to canavanine conferred by *tor2-ts6* was likely due to a defect in uptake because the *tor2-ts6 leu1-32* mutant, whose growth was dependent on leucine supplemented in synthetic media, grew poorly on EMM media containing leucine at a concentration of 40 μg/ml (Y. NAKASE, unpublished result).

The crosses between the *tor2-ts6* mutant and the *rhb1-DA8* mutant produced tetrads in which only two or three spores were viable (Figure 7B). We determined the genotype of each viable spore by sequence analysis to identify the *rhb1-DA8* mutation and by testing the temperature sensitivity to identify the *tor2-ts6* mutation. We could thereby deduce the genotype of the nonviable spores. The results indicated that all the nonviable spores were double mutants (*tor2-ts6 rhb1-DA8*). Other tetrads produced four viable spores, two of which were *tor2-ts6* and the other two of which were *rhb1-DA8* (not shown). We thus concluded that the *rhb1-DA8* mutation caused a synthetic lethality with the *tor2-ts6* mutation. The crosses between the *tor2-ts10* mutant and the *rhb1-DA8* mutant produced double mutants (*tor2-ts10 rhb1-DA8*), which could occasionally form tiny colonies after incubation for >10 days. We, however, failed to isolate a double-mutant strain due to spontaneous reversion (not shown). These results indicated that the *rhb1-DA8* allele produced a synthetic lethal or sick phenotype when combined with the *tor2-ts* alleles. *Rhb1-DA8* could not normally stimulate Tor2.

Because we could not construct a double mutant (*rhb1-DA8 tor2-ts6*), the *rhb1-DA8* mutation was introduced ectopically into the *tor2-ts6* mutant by transformation. As shown in Figure 7C, upon introduction of the *rhb1-DA8* gene into the *tor2-ts6* mutant, the mutant became more resistant to canavanine. Similarly, the *rhb1-DA4* gene conferred a stronger resistance to the drug when it was introduced ectopically into the *tor2-ts6* mutant.

We finally examined the functional relationship between Tor1 and Rhb1. As shown in Figure 7D, the resistance to canavanine conferred by *rhb1-DA4*, *rhb1-DA8*, or $\Delta tsc2$ was not fully suppressed by $\Delta tor1$, indicating that Tor1 does not directly interact with Rhb1 to cause the resistance to the drug.

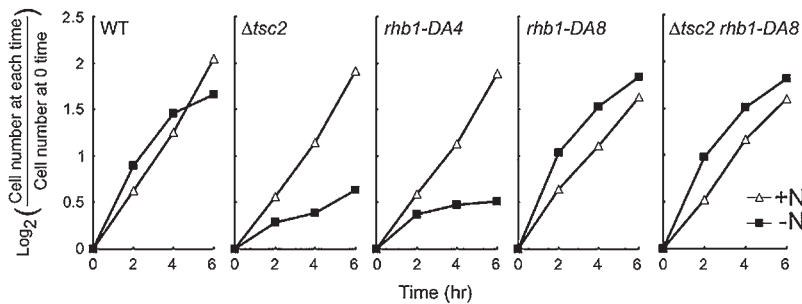


FIGURE 4.—Rate of cell division after nitrogen starvation. Wild-type cells (972h⁻), $\Delta tsc2$ cells (AE509), *rhb1*-DA4 cells (M5-2g3), *rhb1*-DA8 cells (M21-13), and $\Delta tsc2$ *rhb1*-DA8 cells (M47-25) were grown at 26° with or without a nitrogen source, and a rate of cell division was determined. Δ , EMM with a nitrogen source (93.5 mM NH₄Cl); ■, EMM without nitrogen source.

DISCUSSION

In this study, we generated two dominant active *rhb1* mutants, *rhb1*-DA4 and *rhb1*-DA8, and characterized them genetically in fission yeast. Although the two mutants were isolated through the same genetic screen, they responded to nitrogen starvation quite differently.

Two dominant active mutants, *rhb1*-DA4 and *rhb1*-DA8: In the *rhb1*-DA4 mutant, valine at position 17 is replaced with alanine. Ras and other small GTPases are built with several conserved functional domains, namely, G1, G2, G3, G4, and G5 boxes (BOURNE *et al.* 1991). The V17A mutation is located in the G1 box (Figure 1B) that contacts phosphates of GTP or GDP. This mutation (V17A) and a similar one (V17G) in fission yeast Rhb1 GTPase were also identified previously in a screen similar to that reported in this study. It was demonstrated that the mutation of V17G abolishes the guanine nucleotide binding (URANO *et al.* 2005). Activating mutations in budding yeast Ras2 at an analogous position such as V21D and V21G were also shown to abolish the nucleotide binding (DALLEY and CANNON 1996). Our analysis in this study indicated that the *rhb1*-DA4 mutation mimics $\Delta tsc2$, in which Rhb1 GTPase would remain as a GTP-bound form due to loss of the Tsc2-dependent GAP activity. Taken together, we speculate that *rhb1*-DA4 can stimulate its downstream elements regardless of the guanine-nucleotide-binding status.

The *rhb1*-DA8 carries two mutations replacing glutamine at position 52 with arginine (Q52R) and isoleucine at position 76 with phenylalanine (I76F). The amino acids at these positions are conserved in human Rheb (Figure 1B). Of the two mutations in *rhb1*-DA8, I76F is located within switch II, a domain considered to be important for interaction with GAP and effectors (VETTER and WITTINGHOFFER 2001; YU *et al.* 2005). A recent study of human Rheb has demonstrated that a double mutation of I76A D77A almost completely abolishes the ability to stimulate its effector, mTOR, although the mutation does not affect binding to mTOR (LONG *et al.* 2007). These results would suggest that the I76F mutation in *rhb1*-DA8 might affect the ability of Rhb1 GTPase to stimulate the Tor2 kinase. While the *rhb1*-DA8 mutant exhibits strong resistance to canavanine, it allows response to nitrogen starvation. The

mutant divides without net growth and allows induction of the *mei2*⁺ gene upon the shift to the nitrogen-free media. Because these events that are adaptive to nitrogen starvation are normally suppressed by the Tor2 kinase, the phenotype of the *rhb1*-DA8 mutant would also suggest a weak activity of Rhb1-DA8 GTPase to stimulate Tor2. More directly, the *rhb1*-DA8 mutation causes a synthetic lethality with the *tor2-ts6* mutation (Figure 7B). On the basis of the results from our genetic analysis and the preceding biochemical and structural studies of human Rheb, it is likely that the Rhb1-DA8 GTPase is partially defective in activating the Tor2 kinase.

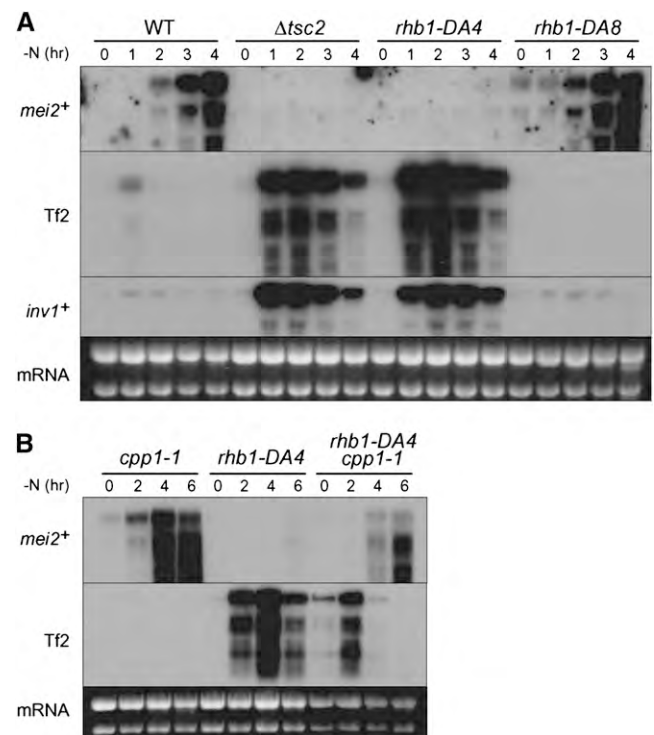


FIGURE 5.—Expression of *mei2*⁺ and Tf2. (A and B) Wild-type cells (972h⁻), $\Delta tsc2$ cells (AE509), *rhb1*-DA4 cells (M5-2g3), *rhb1*-DA8 cells (M21-13), *cpp1-1* cells (YKK90), and *rhb1*-DA4 *cpp1-1* cells (M7-1) were precultured overnight in EMM containing 93.5 mM NH₄Cl as a nitrogen source and then transferred to EMM lacking a nitrogen source at 30°. Total RNAs were analyzed by Northern blot with the *mei2*⁺ gene, *inv1*⁺ gene, and Tf2 as probes.

TABLE 2
Gene expression profile after nitrogen starvation

ORF ID	Gene name	Possible function
Group A: Genes induced more than fourfold in both wild type and <i>rhb1-DA8</i> , but not in $\Delta tsc2$ and <i>rhb1-DA4</i>		
SPAC27D7.03c	<i>mei2</i>	RNA-binding protein involved in meiosis
SPAC1039.03		Esterase/lipase
SPAC186.04c		Pseudogene
Group A': Genes induced more than twofold in both wild type and <i>rhb1-DA8</i> , but not in $\Delta tsc2$ and <i>rhb1-DA4</i>		
SPCC550.07		Acetamidase
SPCC550.10	<i>meu8</i>	Aldehyde dehydrogenase
SPAC186.04c		Pseudogene
SPAC19G12.16c	<i>adg2</i>	Conserved fungal protein
SPBC3E7.12c	<i>chr1</i>	Chitin synthase regulatory factor
SPAC13G7.04c	<i>mac1</i>	Membrane anchored protein
SPAC3F10.15c	<i>spo12</i>	Spo12 family protein
SPBC16D10.08c		Heat-shock protein Hsp104
SPAC4G9.19		DNAJ domain protein DNAJB family
SPAC1142.03c	<i>swi2</i>	Swi5 complex subunit
SPAC16A10.04	<i>rho4</i>	Rho family GTPase
SPAC23C11.16	<i>plol1</i>	Polo kinase
SPAC25B8.08		Conserved fungal protein
SPAC167.07c		Ubiquitin-protein ligase E3
SPAC11E3.13c	<i>gas5</i>	1,3- β -Glucanosyltransferase
SPBC3H7.03c		2-Oxoglutarate dehydrogenase
SPAC458.05	<i>pik3</i>	Phosphatidylinositol 3-kinase
SPAC26F1.01	<i>sec74</i>	Guanyl-nucleotide exchange factor
SPBPB7E8.01		Sequence orphan
SPAC18G6.01c		Calchone-related protein family
SPAC9.10	<i>thi9</i>	Thiamine transporter
SPAC1F7.03	<i>pkd2</i>	Tryptophan-like ion channel
SPAC6F12.12	<i>par2</i>	Protein phosphatase regulatory subunit
SPBC2F12.05c		Sterol-binding ankyrin repeat protein
SPBC32F12.11	<i>tdh1</i>	Glyceraldehyde-3-phosphate dehydrogenase
Group B: Genes induced more than fourfold in both $\Delta tsc2$ and <i>rhb1-DA4</i> , but not in wild type and <i>rhb1-DA8</i>		
SPCC191.11	<i>inv1</i>	β -Fructofuranosidase
SPCC70.08c		Methyltransferase
SPAC3C7.02c		Protein kinase inhibitor
SPBC1271.08c		Sequence orphan
SPAC21E11.04	<i>ppr1</i>	L-Azetidine-2-carboxylic acid acetyltransferase
SPCC70.10		Sequence orphan
SPAC2E1P3.03c	Tf2-3	Retrotransposable element
SPCC1494.11c	Tf2-13-pseudo	Retrotransposable element
SPBC2G2.04c	<i>mmf1</i>	YjgF family protein
SPAC167.08	Tf2-2	Retrotransposable element
SPBC1E8.04	Tf2-10-pseudo	Retrotransposable element
SPBC9B6.02c	Tf2-9	Retrotransposable element
SPAPB15E9.03c	Tf2-5	Retrotransposable element
SPAC821.10c	<i>sod1</i>	Superoxide dismutase
SPAC9.04	Tf2-1	Retrotransposable element
SPCC1020.14	Tf2-12	Retrotransposable element
SPAC26A3.13c	Tf2-4	Retrotransposable element
SPAC25B8.09		Trans-aconitate 3-methyltransferase

ORFs that were induced more than fourfold (in group A) or twofold (in group A') in both the wild-type strain (972h⁻) and the *rhb1-DA8* mutant (M21-13), but not in the $\Delta tsc2$ (AE509) and the *rhb1-DA4* mutant (M5-2g3), 3 hr after nitrogen starvation at 30°, are listed. ORFs induced more than fourfold in both $\Delta tsc2$ and the *rhb1-DA4* mutant, but not in the wild-type strain and the *rhb1-DA8* mutants, are listed in group B. Microarray analysis was performed twice, and we list here only ORFs for which the result was reproducibly obtained. The DNA microarray data were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/index.cgi>) under accession no. GSE14626.

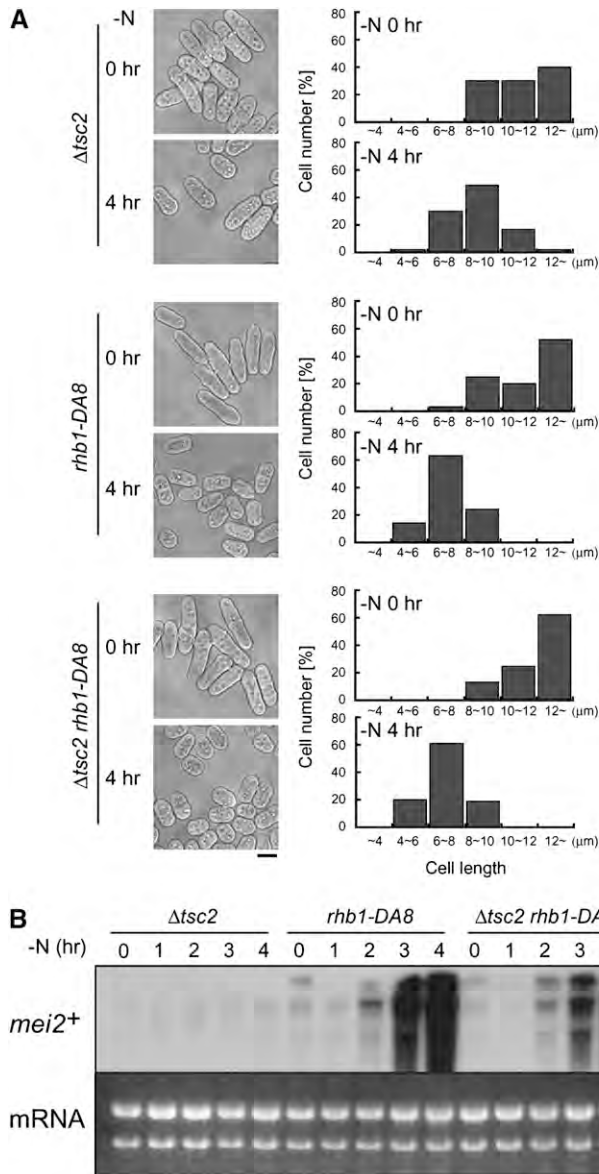


FIGURE 6.—Suppression of $\Delta tsc2$ by *rhb1-DA8*. (A) $\Delta tsc2$ cells (AE509), *rhb1-DA8* cells (M21-13), and $\Delta tsc2$ *rhb1-DA8* cells (M47-25) were examined for morphology and cell length as in Figure 3. (B) Each strain was examined for expression of the *mei2⁺* gene as in Figure 5.

Roles of the Tor2 kinase in uptake: It was previously shown that overexpression of *tor2⁺* caused a resistance to canavanine in a wild-type strain, but not in a strain deleted for the *tor1⁺* gene (WEISMAN *et al.* 2007). Activation of Tor2 kinase may thereby inhibit uptake of amino acids in a *tor1⁺*-dependent manner. In this study, we found that a temperature-sensitive allele of *tor2⁺*, *tor2-ts6*, was defective in uptake of leucine and conferred a resistance to canavanine. The two results are seemingly inconsistent.

In the fission yeast genome, ~20 amino acid permeases are encoded, of which 11 permeases are activated at the transcriptional level upon nitrogen

starvation. On the other hand, 5 are unchanged or even inactivated (Sanger Center Gene DB <http://www.genedb.org/genedb/pombe/index.jsp> and NAKASE *et al.* 2006). It is likely that some permeases are required when the cell is starved for nitrogen. Other permeases, whose expression is not influenced by the availability of nitrogen, are responsible for uptake under actively growing conditions. The Tor2 kinase, as an essential component for growth, may be responsible for activating a set of permeases under actively growing conditions. A partial loss of function of *tor2⁺* would thereby result in reduction of a total activity of uptake and consequently confer a resistance to canavanine. It is also possible that the Tor2 kinase may be responsible for regulation of a set of permeases, which are expressed at a basal level under actively growing conditions, but derepressed upon nitrogen starvation. Overexpression of *tor2⁺* would result in suppression of the activity of these permeases and thereby confer a resistance to canavanine. Indeed, expression two permeases, Isp5 and 7G5.06, have been shown to be repressed in $\Delta tsc2$, a genetic background in which Tor2 kinase would be activated (WEISMAN *et al.* 2007).

The mechanism to control the uptake of amino acids is still to be elucidated on the molecular basis. In addition to regulation at the transcriptional level, the total activity of the uptake is likely regulated by stability and/or localization of individual amino acid permeases according to the nutrient condition. It was demonstrated recently that a loss of *tsc2⁺* ($\Delta tsc2$) caused mislocalization of Cat1, a cationic amino acid permease, and that a mutation in the *pub1⁺* gene coding a ubiquitin ligase suppressed the mislocalization in $\Delta tsc2$ (ASPURIA and TAMANOI 2008). Pub1 would be a key component in determining the activity of the uptake at the post-translational level. Its functional relationship with Rhb1 and Tor2 is to be elucidated in the future.

Signaling by Rhb1 GTPase: While amino acid uptake was prevented by both *rhb1-DA4* and *rhb1-DA8* in a dominant fashion, the response to nitrogen starvation was prevented only by *rhb1-DA4*. *rhb1-DA8* thereby allows genetic dissection of the Rheb-dependent signaling cascade.

Although both overexpression of *tor2⁺* and the *rhb1-DA8* mutant causes resistance to canavanine, we speculate that they cause the resistance to the drug by independent mechanisms for the following reasons: (1) the synthetic lethality of the *rhb1-DA8 tor2-ts6* double mutant suggests that the *rhb1-DA8* mutant activates the Tor2 kinase only partially, and (2) the *rhb1-DA8* mutant, unlike overexpression of the *tor2⁺* gene, confers the resistance to canavanine independently from the function of Tor1. In addition, resistance to canavanine exhibited by a particular mutant could indicate that the mutant is tolerant to non-native proteins that have misincorporated canavanine. In this respect, we should not uniformly consider mutants resistant to the drug to be defective in uptake of amino acids.

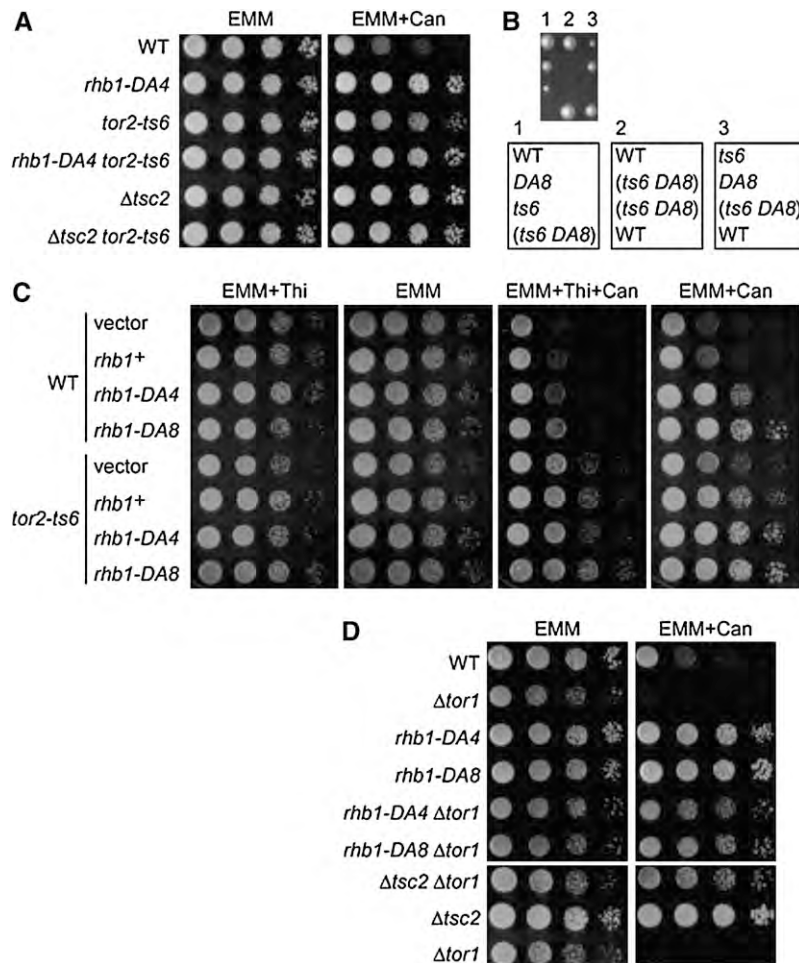


FIGURE 7.—Interaction between Rhb1 and Tor. (A) Wild-type cells (972h⁺), *rhb1-DA4* cells (M5-2g3), *tor2-ts6* cells (YKK282), *rhb1-DA4 tor2-ts6* cells (M86-20), $\Delta tsc2$ cells (AE509), and $\Delta tsc2 tor2-ts6$ cells (YKK278) were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test as in Figure 2A. The plates were incubated 26° for 3 days without canavanine and 5 days with canavanine. (B) The genotype of each spore in the tetrads produced by a cross between *tor2-ts6* (YKK282) and *rhb1-DA8* (M21-13) was determined for viable spores and deduced for nonviable ones (in parentheses). (C) Wild-type cells (SP6) and *tor2-ts6* cells (YKK273) carrying pREP81, pREP81-*rhb1*⁺, pREP81-*rhb1-DA4*, or pREP81-*rhb1-DA8* were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test as in Figure 1A. The plates were incubated at 26° for 3 days without canavanine and 5 days with canavanine. (D) Wild-type cells (972h⁺), $\Delta tor1$ cells (M87-18), *rhb1-DA4* cells (M5-2g3), *rhb1-DA8* cells (M21-13), *rhb1-DA4 Δtor1* cells (M87-7), *rhb1-DA8 Δtor1* cells (M88-23), $\Delta tsc2 \Delta tor1$ cells (YKK418), and $\Delta tsc2$ cells (AE509) were examined for their resistance to canavanine at a concentration of 60 μ g/ml by spot test as in Figure 2A. The plates were incubated at 26° for 3 days without canavanine and 5 days with canavanine.

The signaling cascade would branch below the Rhb1 GTPase or the Tor2 kinase. If it branches just below the Rhb1, the Rhb1 GTPase may regulate Tor2 and an element yet to be identified. Tor2, when stimulated by the Rhb1 GTPase, is responsible for suppressing the events adaptive to nitrogen starvation. The other one would be responsible for controlling amino acid uptake. While Rhb1-DA4 would constitutively stimulate both of these elements, Rhb1-DA8 would largely stimulate the one controlling the uptake. We demonstrated in this study that the Rhb1-DA8 GTPase could not stimulate Tor2 even in the $\Delta tsc2$ background, a condition under which the Rhb1-GTPase would exist as a GTP-bound form. Thus, the ability of the Rhb1-DA8 GTPase to stimulate its downstream elements may not be influenced by the guanine-nucleotide-binding status. It is rather likely that the Rhb1-DA8 GTPase may have an altered specificity toward its downstream elements and preferentially stimulate the one controlling the uptake of amino acids.

It is equally possible that the signal cascade branches below Tor2. The Tor2 kinase might exist as at least two different forms: one to support cell growth and suppress the response to nitrogen starvation and the other one to regulate the activity of a particular set of amino acid permeases at the transcriptional, translational, or post-

translational level. Rhb1-DA8 may specifically stimulate the later form of Tor2 and reduce a total activity of the amino acid uptake. A partial loss of Tor2 suppresses a defect in the uptake of adenine caused by $\Delta tsc2$ (MATSUO *et al.* 2007). Another form of Tor2 might be involved in the uptake of nucleotides.

Tuberous sclerosis complex pathology: Mutations in the human genes *TSC1* and *TSC2* are predisposing to a disease, tuberous sclerosis complex (TSC) (EUROPEAN CHROMOSOME 16 TUBEROUS SCLEROSIS CONSORTIUM 1993; VAN SLEGTENHORST *et al.* 1997). Because we showed that the *rhb1-DA4* mutation mimics deletion of the *tsc2*⁺ gene in fission yeast, it is plausible that an analogous mutation in human Rheb would cause a symptom similar to that found in patients with TSC. Human Rheb could be a third TSC gene. Because our model study in fission yeast demonstrated that the phenotypes significantly varied depending on the allele, the symptoms of the Rheb-induced TSC could also be variable.

We thank M. Yamamoto (University of Tokyo, Tokyo, Japan) for strains. This work was supported by a grant from U. S. Army Medical Research and Materiel Command "Tuberous Sclerosis Complex Research Program" (to T. Matsumoto), by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.H.), and by a grant from the Japan Society for the Promotion of

Science (JSPS) (to Y.C.). T. Murai was a recipient of a scholarship from Teijin (Osaka, Japan) and a predoctoral fellowship from JSPS. Y.N. was supported by an exploratory research grant from JSPS.

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