AD_____

Award Number: W81XWH-05-1-0164

TITLE: A Genetic Approach to Define the Importance of Rheb in Tuberous Sclerosis

PRINCIPAL INVESTIGATOR: Fuyuhiko Tamanoi, Ph.D.

CONTRACTING ORGANIZATION: University of California Los Angeles
Los Angeles, CA 90095-1489

REPORT DATE: January 2008

TYPE OF REPORT: Final Addendum

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2008	Final Addendum	15 DEC 2006 - 14 DEC 2007

4. TITLE AND SUBTITLE

A Genetic Approach to Define the Importance of Rheb in Tuberous Sclerosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0164

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Fuyuhiko Tamanoi, Ph.D.

E-Mail: fuyut@microbio.ucla.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California Los Angeles
Los Angeles, CA 90095-1489

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
This report summarizes our accomplishments in characterizing the TSC/Rheb/mTOR signaling pathway that is altered in tuberous sclerosis. We have generated mice with decreased expression of Rheb1. We have succeeded in raising an antibody against mouse Rheb2. Effects of the TSC/Rheb/mTOR signaling on cell cycle progression have been investigated and we have obtained results suggesting the involvement of p27 and AMPK. Novel activating mutations of mTOR have been identified and they were used to investigate the consequences of the activation of the TSC/Rheb/mTOR signaling pathway on cell physiology. Our study makes significant contribution to understand how the TSC/Rheb/mTOR signaling pathway is regulated. The results we obtained make important contribution to the understanding of tuberous sclerosis.

15. SUBJECT TERMS
No subject terms 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	15	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction…….... 4

BODY……. 5-9

Key Research Accomplishments…… 9

Reportable Outcomes…….. 9

Conclusions…….. 10

References…… 10

Appendices……. Yes

Appendix 1: Urano, J., Sato, T., Matsuo, T., Otsubo, Y., Yamamoto, M. and Tamanoi, F. (2007) Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mTOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* 104, 3514-3519.

Appendix 2: Miyamoto, S., Kato-Stankiewicz, J. and Tamanoi, F. (2005) The regulation of cell cycle progression by Tsc and Rheb GTPase. *Proceedings American Association for Cancer Research*, Volume 46, Abstract #5434

Introduction

This is the final report for the project that covers the period December 15, 2004 – January 14, 2008. Tuberous sclerosis is caused by mutations in the *Tsc1* or *Tsc2* gene. Products of these genes form a complex that acts as a negative regulator of Rheb GTPase, an activator of mTOR. Thus, one of the major problems with tuberous sclerosis is that the TSC/Rheb/mTOR signaling pathway is over-activated. Our research is focused on understanding how this signaling pathway is regulated and what the consequences of alteration of this signaling pathway are. We have accomplished most of the tasks described in our Statement of Work. We have generated mice with decreased Rheb1 expression. Rheb2 specific antibody has been raised and was used to characterize Rheb2. We have characterized altered cell cycle progression in the *Tsc*-null MEFs. We have generated novel mTOR mutants and examined consequences of the activation of the TSC/Rheb/mTOR signaling.

Summary

Tuberous sclerosis is caused by the loss of Tsc1/Tsc2 complex that acts as a negative regulator of Rheb GTPase [1]. This results in overactivation of mTOR causing uncontrolled growth. The overall aim of this grant was to understand the consequences of altering this signaling pathway. During the funding period, we have made a number of progresses including (i) generation of mice with decreased expression of Rheb1, (ii) identification and characterization of Rheb2 by raising Rheb2 specific antibody, (iii) elucidation of how the overactivation of the Tsc/Rheb/mTOR signaling results in altered cell cycle progression, (iv) generation of constitutive active mutant forms of mTOR that can bypass amino acid requirement for their activation.

Detailed description of accomplishments

Task 1: To generate and characterize Rheb-knockout mice

To generate *Rheb1* knockout mice, we have designed and constructed a knockout vector. This targeting vector was designed to replace exon 2 of the mouse *Rheb1* gene with a neomycin phosphotransferase cassette. The vector was used to generate *Rheb1* (-/-) homozygous embryonic stem (ES) cells. This was confirmed by southern blot using a 3' genomic probe. *Rheb1* (+/-) as well as (-/-) mice were then generated using these ES cells. Genomic DNA from the tail of these mice was analyzed which demonstrated that a fragment expected from the *Rheb1* knockout sample is detected (Figure 1).

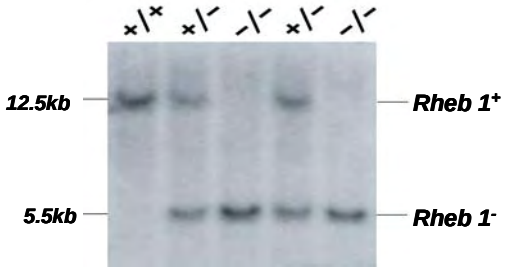

Fig.1. Identification of Rheb1$^{-/-}$ mice. Genomic DNA from mouse tails was digested with Kpn I and probed with a 3' flanking probe.

To examine whether *Rheb1* gene product is missing in the knockout mice, we evaluated the amount of Rheb1 message by RT-PCR. RNA was isolated from the +/+ and -/- animal tails, reverse transcribed to yield cDNA which was then amplified by PCR using Rheb1 specific primers. Significant differences in the amount of the Rheb1 message were detected between the +/+ and -/- samples (Figure 2A). Northern blot was also applied to compare the amount of *Rheb1* message. This was carried out by isolating RNA from the +/+ and -/- mice (tail). RNA samples were loaded on a gel and probed with a Rheb1 specific probe. In this experiment, GAPDH message was used as a loading control. As can be seen in Figure 2B, the amount of *Rheb1* message in the sample from the -/- animal was significantly decreased compared with that from the +/+ animal. Although the amount of *Rheb1* message was decreased in the -/- mice, it was not completely missing.

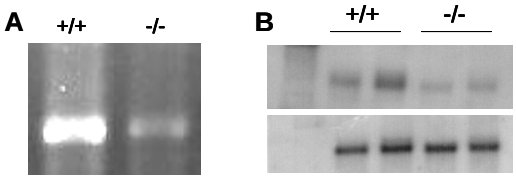

Fig.2. A: RT-PCR of RNA isolated from Rheb1$^{+/+}$ and Rheb1$^{-/-}$ mice tails using Rheb1 specific primers. B: RNA from Rheb1+/+ and Rheb1-/- mice tails were probed with a Rheb1 probe (upper panel). Lower panel shows GAPDH expression used as a loading control.

Further analysis suggested that the -/- mice we generated contain insertion of the neo cassette rather than gene replacement we set out to accomplish. This appears to explain why the *Rheb1* message was not completely missing. Our conclusion is based on the analysis of the *Rheb1* gene alteration in the -/- mice. Tail DNA samples from the +/+, +/- as well as -/- mice were analyzed for the presence of exon 2 by PCR. The fragment expected to be obtained by the amplification of exon 2 was detected in the -/- as well as in the +/- mouse samples. The *Rheb1*-/- mice we generated did not exhibit growth defects or any altered phenotypes. Based on the above observations, it is possible that the small amount of Rheb1 we detected was sufficient to provide the function of this protein.

To generate Rheb knockout mice that represent gene replacement, we tried again. We consulted with Dr. Michele Musacchio at the University of California Irvine knockout mouse facility. The targeting constructs for Rheb1 and Rheb2 were electroporated into mouse ES cells and the cells with correct chromosomal replacement were screened by Southern hybridization. After extensive screening of ES cells, we failed to identify knockout ES cells for either Rheb1 or Rheb2.

Task 2: To examine the effects of Rheb inhibition on tumor incidence observed with heterozygous Tsc-knockout mice.

As discussed above, the mice we generated were not the ones carrying gene replacement we expected but were most likely insertion of the knockout vector. Thus, even though there is significant decrease of the expression of Rheb1, there is still a residual level of Rheb1. We made decision at that point not to pursue this task, as the results will be ambiguous and not easy to interpret. Instead, we decided to pursue experiments described in Tasks 4-7.

Task 3: To examine if Rheb-knockout mutations prevent the developmental lethal phenotype of homozygous Tsc-knockout mice.

We faced a decision similar to that described in Task 2 above. Again, we decided against pursuing this task, as the results obtained will not be easily interpreted. Instead, we decided to focus on experiments described in Tasks 4-7.

Task 4: To characterize Rheb2

We have been successful in raising a polyclonal antibody against Rheb2 (RhebL1). The antibody was raised using a 14-residue C-terminal peptide spanning from amino acid 167 to 180. This sequence was chosen, as there is no amino acid identity between Rheb1 and Rheb2 within this sequence. The antibody was prepared by Washington Biotechnology (Maryland) using two New Zealand rabbits. The antibody was affinity purified using Rheb1 column.

Characterization of the antibody established that the antibody is specific to Rheb2 and does not detect mouse Rheb1 (Figure 3). Although Rheb1 antibody with improved potency has been developed over the years, this is the only antibody that is specific to Rheb2. We also found that the antibody is specific to mouse Rheb2 protein, as it does not recognize human Rheb2 (Figure 3).

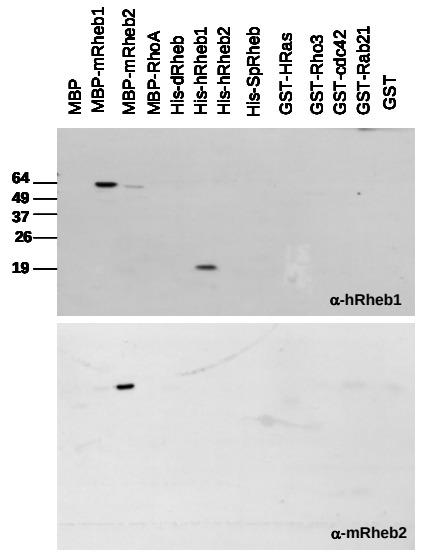

Fig.3. Specificity of anti-mRheb2 antibody. Reactivity of anti-mRheb2 antibody against a variety of proteins is shown in the lower panel. The upper panel shows reactivity of anti-Rheb1 antibody.

Using the Rheb2 antibody, we have characterized Rheb2. A band of Rheb2 was detected in extracts from various mouse cell lines including 3T3-L1 pre-adipocytes, L1C2 lung carcinoma cells and PC12 (pheochromocytoma) cells. To examine tissue expression of Rheb2, mouse tissue samples including brain, heart, kidney, liver, testis and muscle were prepared and probed with the Rheb2 antibody. We found that Rheb2 is highly expressed in the brain. This is interesting and supports the idea that Rheb2 expression is non-ubiquitous. Similar non-ubiquitous expression of human Rheb2 was recently reported [2,3]. Therefore, while Rheb1 is expressed ubiquitously, Rheb2 appears to exhibit tissue specific expression.

Task 5: To investigate the mechanism of regulation of cell cycle progression by the TSC/Rheb/mTOR signaling pathway

Characterization of MEFs derived from Tsc-null mice:

One of the consequences of the activation of the TSC/Rheb/mTOR signaling pathway is failure to block cell cycle progression upon nutrient starvation. On the other hand, inhibition of this signaling pathway results in cell cycle block at the G0/G1 phase. We have shown this point by using *Drosophila* tissue culture cell line S2 and inhibiting Rheb expression by using siRNA against Rheb [4]. This observation was further investigated using MEFs derived from *Tsc*-null mice. These cells do not respond to serum starvation and continue growing even in the absence of serum. In addition, growth inhibition was not observed even after the cells reached high density. Since cell cycle progression from G1 to S is regulated by the activity of Cdk2, we examined Cdk2 activity by immunoprecipitating Cdk2 and assaying its kinase activity by using histone H1 as a substrate. As shown in Figure 4, Cdk2 activity remains high even after serum starvation in the *Tsc2*-null MEFs. Similarly, Cdk2 activity remains high after the *Tsc2*-null MEF cells reached high confluency. This contrasts with Cdk2 immunoprecipitated from the control parental MEFs; the level of Cdk2 is decreased after serum starvation.

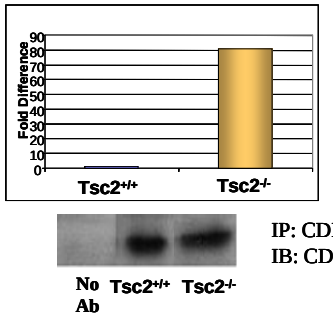

Fig.4. Cdk2 is constitutively activated in Tsc-null MEFs. Kinase activity of Cdk2 immunoprecipitated from cells after serum starvation. The lower panel shows that comparable amounts of Cdk2 were recovered.

Characterization of cell cycle proteins

We have examined the level of cell cycle proteins to gain insight into the mechanism of cell cycle alteration in the *Tsc2*- null MEF. We first found that the levels of Cdk2 and Cdk4 are unchanged in the *Tsc2*-null MEF with or without serum starvation. We also did not see change in the level of Cdk inhibitors, p27 and p21. On the other hand, the level of p16 is increased. We also found that the level of cyclin D is increased in the *Tsc2*-null MEF.

Further investigation into the Cdk inhibitor, p27 revealed that there is consistent and significant difference in the nuclear localization of p21 between the *Tsc2*-null MEF and control MEF. While nuclear translocation of p27 is observed after serum starvation in the control MEF, p27 is not detected in the nuclear fraction in the *Tsc2*-null MEF. To further characterize this observation, we collaborated with Dr. Cheryl Walker (MD Anderson Cancer Center). We found that the nuclear translocation of p27 is dependent on its phosphorylation by AMPK. In addition, this study identified the sites of AMPK phosphorylation on p27.

Task 6: To elucidate mechanisms that result in the activation of the TSC/Rheb/mTOR signaling pathway

Activating mutations of mTOR

We have identified activating mutations of mTOR. We found that changing leucine to proline at residue-1460 of mTOR confers constitutive activation of this kinase. This mutation occurs within the FAT domain. Likewise, mutating glutamine at residue-2419 to lysine confers similar constitutive activation. This mutation occurs in the kinase domain. Since mTOR activity is dependent on the presence of nutrients such as amino acids, mTOR activity is low when cells are amino acid starved. Figure 5 shows the results obtained by examining phosphorylation of S6 as well as by assaying kinase activity of mTOR immuneprecipitates. While transfection of the wild type mTOR did not rescue nutrient starvation, significant level of mTOR activity was detected when constitutive active mTOR mutants were transfected, suggesting that these mutants confer amino acid independent growth.

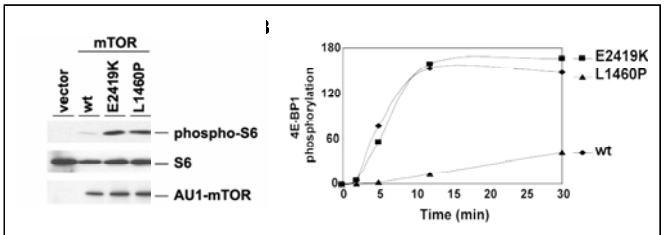

Fig.5. Cells (HEK293) transfected with constitutively active mTOR mutants exhibit high level of phospho-S6 even after nutrient starvation (Left panel). Kinase activity in vitro is also constitutively active (Right panel).

The above mTOR mutations were originally identified by our work on fission yeast Tor2. This mTOR homolog forms a complex called TORC1 and is responsible for growth and cell cycle regulation of fission yeast. TORC1

requires Rheb for its function. A genetic screen was devised to identify mutant forms of Tor2 that can bypass dependency on Rheb for growth. In addition, another screen based on mating inhibition was carried out. Twenty two different single amino acid changes were identified that confer Rheb independent growth. Clustering of these mutations in two regions, one in the FAT domain and the other in the kinase domain, was revealed.

Two types of mTOR complexes are present in mammalian cells; mTORC1 contains mTOR, Raptor and mLST8 and is involved in growth control mediated by the stimulation of protein synthesis, while mTORC2 contains mTOR, Rictor and mLST8 and is responsible for the phosphorylation of Akt. Our results showed that the activating mTOR mutations affect mTORC1 but not mTORC2.

Characterization of mTOR

We have further characterized mTOR mutants. Presence of associated proteins was examined by immunoprecipitating mTOR. We found that comparable levels of Raptor, Rictor and mLST8 were associated with the constitutively active mTOR compared with the wild type protein, suggesting that the mutations do not affect the overall structure of the mTOR complex.

We also found that the activating mutations exert dominant effects. Existence of mTOR dimer was demonstrated by using two different tags AU1 and FLAG. AU1-tagged mTOR and FLAG-tagged mTOR were co-expressed. Immunoprecipitation of AU1 mTOR showed that FLAG-mTOR also came down in the immuneprecipitates. We then constructed AU1 tagged mutant mTOR and co-expressed it with FLAG tagged wild type mTOR. Immunoprecipitation of FLAG-tagged mTOR showed that AU1-mTOR is coprecipitated, suggesting that a heterodimer is formed. Examination of this heterodimer showed that it is active even in the presence of mutant mTOR. We believe that this observation is important, as heteroygous mutations could result in constitutive activation of mTOR.

mTOR constitutive active mutants are rapamycin sensitive

Another important observation we made concerns rapamycin that is evaluated in clinics as anticancer drugs. As shown in Figure 6, rapamycin inhibited constitutively activated mTOR. In this experiment, the wild type and two different mutants of mTOR were transfected into cells. The cells were nutrient starved and then treated with rapamycin. Phosphorylation level of S6 was examined to assess the activity of mTOR. The results suggest that the activity of the mutant mTOR can still be inhibited by rapamycin.

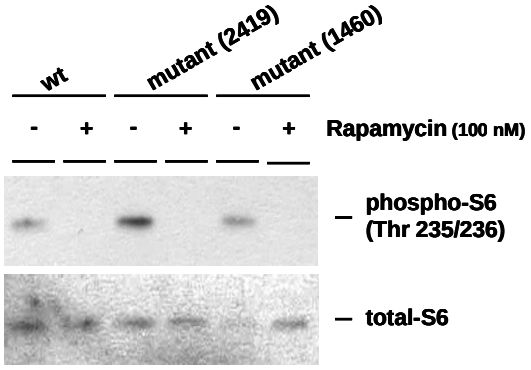

Fig.6. The wild type as well as mutant mTOR activities were inhibited were rapamycin.

Task 7: To examine consequences of activation of the TSC/Rheb/mTOR signaling pathway on mammalian cell growth.

To generate stable cell lines expressing constitutive active mTOR (E2419K), we first transfected HEK293 cells with mTOR constructs and selected for cells that expressed mutant mTOR. Stable transformants expressing mTORE2419K were obtained together with control transformants (vector control). This set was used to examine the consequences of mTOR activation.

We first confirmed that the TSC/Rheb/mTOR signaling is constitutively activated. This was shown by subjecting cells to amino acid starvation and examining phosphorylation of S6K and S6 using antibodies specific for phosphorylated forms of these proteins. Phosphorylated forms were identified with the stable transformants expressing the mTOR mutant, while the levels of phospho-S6K and S6 were decreased by amino acid starvation with the control transformants. We have examined stress sensitivity of the transformants. We

found that the stable transformants expressing mTOR mutant exhibit resistance to hydrogen peroxide, while the control cells are sensitive. In contrast, both the mTOR mutant transformants and the control transformants were sensitive to sorbitol. Another observation we made is that both these transformants are sensitive to rapamycin. Thus, the stable transformants we generated provide a valuable reagent to assess the consequences of activation of the TSC/Rheb/mTOR signaling.

Key Research Accomplishments

(1) We generated mice with decreased expression of Rheb1.
(2) We generated polyclonal antibody specific to mouse Rheb2.
(3) We examined tissue expression of Rheb2, and found that the expression was not ubiquitous. This is different from the expression profile of Rheb1.
(4) We found that the activation of the TSC/Rheb/mTOR signaling leads to constitutive activation of Cdk2, a key cell cycle protein functioning at the G1/S phase boundary. We also found that a Cdk inhibitor protein p27 is affected by the activation of the TSC/Rheb/mTOR signaling pathway. Its translocation to the nucleus is blocked.
(5) Novel mutants of mTOR that are constitutive active have been obtained.
(6) We have shown that the expression of these mutants confers constitutive activation of mTOR even in the absence of amino acids.
(7) The activating mutants of mTOR appears not to affect mTORC2 activity.
(8) The activating mutations do not alter binding of mTOR associated proteins.
(9) The activating mTOR mutations exert dominant effects over the wild type protein.
(10) The activated mTOR mutants retain sensitivity to rapamycin.
(11) We have generated a stable cell line expressing activating mTOR mutant.

Reportable Outcomes

(1) Mice with decreased Rheb1 expression were generated.
(2) Rheb2 clones were generated. Rheb disruption construct for targeted gene replacement has been made.
(3) Rheb2 specific antibody was generated.
(4) Constitutive active mutants of mTOR have been generated. These will provide valuable reagents for the study of the Tsc/Rheb/mTOR signaling.
(5) A stable cell line expressing activating mTOR mutant has been generated.

Publications and presentations

Urano, J., Sato, T., Matuso, T., Otsubo, Y., Yamamoto, M. and Tamanoi, F. (2007) Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mTOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* 104, 3514-3519.

Miyamoto, S. (2007) Cell cycle regulation by the TSC/Rheb/mTOR pathway. Master thesis, Dept. of Microbio., Immunol. & Molec. Genet., UCLA

Miyamoto, S., Kato-Stankiewicz, J. and Tamanoi, F. (2005) The regulation of cell cycle progression by Tsc and Rheb GTPase. *Proceedings American Association for Cancer Research,* Volume 46, Abstract #5434

Short, J.D., Houston, K.D., Cai, S., Kim, J., Miyamoto, S., Johnson, C.L., Bergeron, J.M., Broaddus, R.R., Shen, J., Bedford, M.T., Liang, J.T., Tamanoi, J., Kwiatkowski, D.Mills G.D. and Walker, C.L. (2007) Energy Sensing Regulates $p27^{KIP1}$ by AMPK-Mediated Phosphorylation and Cytoplasmic Sequestration, Submitted.

Tamanoi, F. (2007) Gordon Research Conference on Phosphorylation and G-protein mediated signaling networks, Maine

Tamanoi, F. (2007) 2nd Cell Regulations in Division and Arrest Workshop, Okinawa, Japan.

Tamanoi, F. (2006) FASEB Summer Conference on Regulation and Function of Small GTPases, Vermont.

Tamanoi, F. (2006) The LAM Foundation Research Conference, Cincinnati, OH.

Conclusions

We have accomplished most of the task that was outlined in the Statement of Work. They include the following points.

1. Mice with decreased Rheb1 expression were generated.
2. Mouse Rheb2 specific antibody was generated.
3. Tissue specific expression of Rheb2 was observed.
4. Elucidation of the effects of the activation of the TSC/Rheb/mTOR signaling on cell cycle progression.
5. Established the significance of p27 in the cell cycle effects of the TSC/Rheb/mTOR signaling.
6. Identified novel activating mutations of mTOR.
7. The activating mutations confer amino acid independent activation of mTOR.
8. The activating mutations exert dominant effects.
9. The activated mutants retain rapamycin sensitivity.
10. Stable cell line expressing activated mTOR mutant was produced.

These studies should provide important insights into understanding the consequences of altering the TSC/Rheb/mTOR signaling.

References

1. Aspuria, P.J. and Tamanoi, F. (2004) The Rheb family of GTP-binding proteins. Cell Signal. *16*, 1105-1112.
2. Saito, K., Araki, Y., Kontani, K., Nishina, H. and Katada, T. (2005) Novel role of the small GTPase Rheb: Its implication in endocytic pathway independent of the activation of mammalian target of rapamycin. J. Biochem. *137*, 423-430.
3. Yuan, J., Shan, Y., Chen, X., Tang, W., Luo, K., Ni, J., Wan, B. and Yu, L. (2005) Identification and characterization of RHEBL1, a novel member of Ras family, which activates transcriptional activities of NF-kappa B. Molec. Biol. Reports *32*, 205-214.
4. Patel, P.H., Thapar, N., Guo, L., Martinez, M., Maris, J., Gau, C.L., Lengyel, J.A. and Tamanoi, F. (2003) *Drosophila* Rheb GTPase is required for cell cycle progression and cell growth. J. Cell Sci. *116*, 3601-3610

List of personnel receiving pay from the research effort:
Fuyuhiko Tamanoi, PI – 15% effort
Qiaolin Chen, Graduate Student Researcher - 30% effort
Juran Kato – 75% effort prior to departure in March 2006

Appendix 1

PNAS
Proceedings of the National Academy of Sciences of the United States of America www.pnas.org

Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells

Jun Urano, Tatsuhiro Sato, Tomohiko Matsuo, Yoko Otsubo, Masayuki Yamamoto, and Fuyuhiko Tamanoi

PNAS 2007;104;3514-3519; originally published online Feb 20, 2007;
doi:10.1073/pnas.0608510104

Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells

Jun Urano*, Tatsuhiro Sato*, Tomohiko Matsuo†‡, Yoko Otsubo†, Masayuki Yamamoto†, and Fuyuhiko Tamanoi*§

*Department of Microbiology, Immunology, and Molecular Genetics, Jonsson Comprehensive Cancer Center, Molecular Biology Institute, University of California, Los Angeles, CA 90095; and †Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan

Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved December 27, 2006 (received for review September 27, 2006)

Rheb is a unique member of the Ras superfamily GTP-binding proteins. We as well as others previously have shown that Rheb is a critical component of the TSC/TOR signaling pathway. In fission yeast, Rheb is encoded by the *rhb1* gene. Rhb1p is essential for growth and directly interacts with Tor2p. In this article, we report identification of 22 single amino acid changes in the Tor2 protein that enable growth in the absence of Rhb1p. These mutants also exhibit decreased mating efficiency. Interestingly, the mutations are located in the C-terminal half of the Tor2 protein, clustering mainly within the FAT and kinase domains. We noted some differences in the effect of mutation in the FAT domain (L1310P) and in the kinase domain (E2221K) on growth and mating. Although the Tor2p mutations bypass Rhb1p's requirement for growth, they are incapable of suppressing Rhb1p's requirement for resistance to stress and toxic amino acids, pointing to multiple functions of Rhb1p. In mammalian systems, we find that mammalian target of rapamycin (mTOR) carrying analogous mutations (L1460P or E2419K), although sensitive to rapamycin, exhibits constitutive activation even when the cells are starved for nutrients. These mutations do not show significant difference in their ability to form complexes with Raptor, Rictor, or mLST8. Furthermore, we present evidence that mutant mTOR can complex with wild-type mTOR and that this heterodimer is active in nutrient-starved cells.

constitutive active TOR | FAT domain | kinase domain | mating | TORC1

Rheb comprises a unique subfamily of the Ras superfamily of GTP-binding proteins that is conserved from yeast to human and plays important roles in cell growth and cell-cycle regulation (1). We and others have shown that Rheb is an activator of mammalian target of rapamycin (mTOR) and a component of the TSC/mTOR/S6K signaling pathway that regulates protein synthesis in response to growth, energy, and nutrient conditions (1–7). mTOR exists in two distinct protein complexes: mTORC1, which consists of mTOR, Raptor, and mLST8 and is rapamycin-sensitive; and mTORC2, which consists of mTOR, Rictor, mLST8, and mSin1 and is rapamycin-insensitive. mTORC1 is involved in the regulation of translation and cell cycle, and mTORC2 is reported to be involved in actin organization and morphology (8–16). Rheb is down-regulated by a complex consisting of *TSC1* and *TSC2* gene products that act as GTPase activating proteins for Rheb (1–7). Mutations of these genes result in tuberous sclerosis complex, a genetic disorder associated with the appearance of hamartomas in the kidneys, lungs, brain, and skin (17, 18).

In fission yeast, Rheb is encoded by the *rhb1* gene and is essential for growth. Loss of Rhb1p results in small rounded cells arrested with G_1 content of DNA (19, 20). Like mammalian cells, Rhb1p is down-regulated by the Tsc1p/Tsc2p complex (21, 22). Mutations in the *tsc* genes result in a delayed response to nitrogen starvation as well as defects in amino acid uptake (21–25). The fission yeast genome encodes two TOR proteins, Tor1p and Tor2p (26, 27). Although Tor2p, like Rhb1p, is essential for growth, *tor2-*Δ cells are sterile and unable to arrest in G_1 in response to nitrogen starvation (26, 27). Tor1p also is implicated in the regulation of stress response and uptake of some amino acids (26–28). We recently have reported that fission yeast Rhb1p associates with Tor2p (25). Interaction between mammalian Rheb and mTOR also has been reported (29–31).

Because the requirement for Rhb1p for growth in fission yeast likely reflects its vital role in the activation of Tor2p, we speculated that activating mutations of Tor2p may bypass this requirement. Identification of such mutations, which are likely to be constitutive active, is of interest in gaining insight into the mechanism of activation of this kinase. In particular, this study may reveal novel involvement of specific domains in the activation of TOR. The TOR kinases are members of the phosphatidylinositol 3-kinase (PI3 kinase)-related family of kinases and have specific domains, including the HEAT repeats and the FAT, kinase, and FATC domains. Involvement of the HEAT domain in protein–protein interactions, dimerization, and membrane association has been reported (9, 32–38). To date, only one activated TOR mutant has been reported. This mutant, ΔTOR, contains a deletion of the "repressor domain," which includes AMPK (T2446) and S6K (S2448) phosphorylation sites at the C-terminal region of mTOR (39–43). In our study, we carried out a systematic analysis to uncover single amino acid changes that confer constitutive activation of TOR proteins.

We report the identification of 22 different single amino acid changes that confer constitutive activation of Tor2p. Interestingly, the mutations are clustered into two regions: the FAT domain and the kinase domain. Characterization of these mutations showed that they are able to bypass the requirement of Rhb1p for fission yeast growth. The use of these mutants allowed us to observe that Rhb1p also is required for resistance to high salt, high temperature, and toxic amino acid analogs in fission yeast. Furthermore, mTOR carrying analogous mutations exhibited nutrient-independent activity and were able to form mTORC1 and mTORC2. In addition, a heterodimer of wild-type and mutant mTOR also displayed nutrient-independent activity.

Results

Identification of Mutations in Tor2p That Can Bypass Growth Requirement for Rhb1p in Fission Yeast. Rhb1p interacts with Tor2p, and both Rhb1p and Tor2p are essential for growth (19, 20, 25, 27).

Author contributions: J.U., T.S., T.M., Y.O., M.Y., and F.T. designed research; J.U., T.S., T.M., and Y.O. performed research; J.U. and T.S. contributed new reagents/analytic tools; J.U., T.S., T.M., Y.O., M.Y., and F.T. analyzed data; and J.U., T.S., T.M., M.Y., and F.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: mTOR, mammalian target of rapamycin; RIG, Rhb1p-independent growth; FOA, 5-fluoroorotic acid.

‡Present address: Department of Zoology and Animal Biology and National Center of Competence in Research Frontiers in Genetics, University of Geneva, 30 Quai Ernest Ansermet, 1211 Geneva, Switzerland.

§To whom correspondence should be addressed at: Department of Microbiology, Immunology, and Molecular Genetics, 1602 Molecular Sciences Building, 609 Charles E. Young Drive East, Los Angeles, CA 90095-1489. E-mail: fuyu@microbio.ucla.edu.

The article contains supporting information online at www.pnas.org/cgi/content/full/0608510104/DC1.

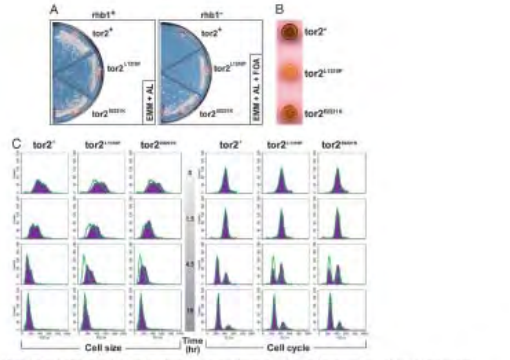

Fig. 1. Tor2p L1310F and E2221K show Rhb1-independent growth and delayed nitrogen starvation response. (*A*) Strains carrying wild-type tor2^{+} (JUp1273) or the activated mutants tor2^{L1310F} (JUp1274) and tor2^{E2221K} (JUp1265) were streaked out onto EMM + AU plates or EMM + AU + FOA plates and incubated at 30°C. EMM, Edinburgh minimal medium. (*B*) JUp1348 (tor2^{+}), JUp1350 (tor2^{L1310F}), and JUp1352 (tor2^{E2221K}) were grown to mid-log phase, washed twice with water, and adjusted to an *A*595 of 1. Aliquots of 3 μl were spotted onto SSA plates, incubated at 25°C for 2 days, and stained by using Iodine vapor. Note that two sets of clones were used, and both show similar results. (*C*) JUp1348 (tor2^{+}), JUp1350 (tor2^{L1310F}), and JUp1352 (tor2^{E2221K}) were grown to mid-log phase, washed twice with water, and resuspended in SSL media. Samples were taken at the indicated times and analyzed with FACS for cell size and cell cycle. Each profile (purple) was overlayed with the profile outline for the wild-type strain (green line) at each time point.

Furthermore, loss of Tor2p function, like the loss of Rhb1p, results in small rounded cells arrested in G_1 (44, 45). Thus, it is likely that in fission yeast, Rhb1p functions to activate Tor2p. This finding raises the possibility that activating mutations in Tor2p (or another downstream factor) can confer Rhb1p-independent growth (RIG). To investigate this point, we have devised a screen to identify yeast mutants that can grow in the absence of Rhb1p [supporting information (SI) Fig. 6]. A strain (JUp1050) was constructed in which the endogenous *rhb1* gene was disrupted by using a *kan*^{R} cassette and growth was maintained by using a wild-type copy of *rhb1*$^{+}$ on a *ura4*$^{+}$-based plasmid. This plasmid can be lost by counterselecting for *ura4*$^{+}$ with 5-fluoroorotic acid (FOA). JUp1050 randomly was mutagenized by methyl-nitro-nitrosoguanidine, and mutants that would grow on media containing FOA (and hence in the absence of *rhb1*$^{+}$) were isolated. To determine whether the *rig* mutation had occurred in *tor2*, we initially sequenced the entire *tor2* ORF.

Approximately 3×10^{8} cells were mutagenized and screened. After eliminating clones that still maintained the *rhb1*$^{+}$ plasmid, a single mutant strain was isolated that was able to grow in the absence of *rhb1*$^{+}$. Sequence analysis of the *tor2* ORF identified a point mutation that results in a glutamate to lysine mutation at position 2221 (E2221K) in the C-terminal half of the Tor2p kinase domain. To confirm that this mutation could confer RIG, this mutation was reintroduced into the *tor2* locus of JUp1050. This strain (JUp1261) was able to grow in the absence of *rhb1* (on FOA), confirming that this mutation in the Tor2p kinase domain confers activity independent of Rhb1p (Fig. 1*A*).

RIG Mutants of Tor2p Exhibit Decreased Mating Efficiency. Because loss of Tor2p function can induce mating (44, 45), we asked whether the Tor2^{E2221K} mutant would exhibit decreased mating. The Tor2^{E2221K} mutation was introduced into a homothallic h^{90} strain, and mating efficiency was assessed by staining with iodine that detects the increased glycogen levels in spores. As can be seen in Fig. 1*B*, we observed a notable decrease in the extent of iodine staining in the mutant. Thus, the E2221K mutation appears to decrease mating efficiency, consistent with an activation of Tor2p.

Additional Tor2 mutations (L1310P, Y1986C, I2229T, and L2233H) were identified by screening for decreased mating (decreased iodine staining) in an h^{90} strain after randomly mutagenizing the *tor2* gene. These mutations were examined for their ability to confer RIG. Fig. 1*A* shows that the strain expressing the L1310P mutant (JUp1274) grew after removing the *rhb1*$^{+}$ plasmid by FOA selection. Similar results were obtained for the other Tor2 mutants. Interestingly, when comparing the L1310P mutant with the E2221K mutant, we noticed that the strain expressing the E2221K mutant has a greater ability to suppress the Rhb1p requirement for growth (see FOA plates in Fig. 1*A*), whereas the L1310P mutant exhibits a more pronounced effect on mating efficiency (Fig. 1*B*). Thus, although RIG and decreased mating are both consequences of a single

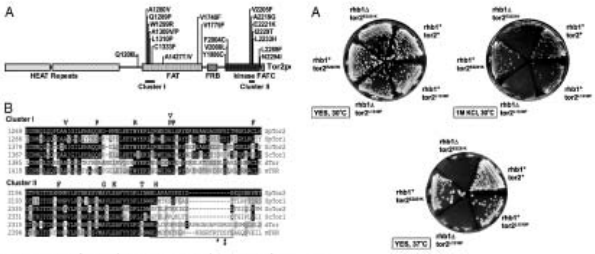

Fig. 2. Location of activated mutations in Tor2p and conservation of residues. (*A*) All identified activating mutations are indicated above the linear representation of Tor2p. Clusters I and II are indicated. (*B*) TOR protein sequences from *Schizosaccharomyces pombe* (SpTor1 and SpTor2), *S. cerevisiae* (ScTor1 and ScTor2), *Drosophila* (dTOR), and human (mTOR) were aligned with MegAlign. Residues identical or similar to SpTor2 are shaded black or gray, respectively. Mutations found in SpTor2 are indicated above the alignment. The repressor domain region of mTOR is indicated by a line under the alignment. Single and double asterisks indicate the AMPK and S6K phosphorylation sites, respectively.

activating mutation in Tor2p, each mutation may affect these two activities differently.

These Tor2 constitutive active mutants exhibit delayed nitrogen-starvation response. Because fission yeast cells respond to nitrogen starvation by arresting in G_1 as small rounded cells, nonauxotrophic strains carrying the L1310P or E2221K mutation were nitrogen-starved in SSL media (see *SI Materials and Methods*), and samples were assessed for cell size and DNA content by FACS. The results are shown in Fig. 1*C*. Forward-scatter analysis shows that both Tor2 mutants show a delay in this change in cell size compared with wild type (notably at 1.5 and 4.5 h). By 10 h, the mutant cells have decreased in size similar to wild-type cells. Analysis of cell-cycle profiles shows that the Tor2 mutants are delayed in the appearance of G_1 cells because more cells are in G_2 at 4.5 h, whereas majority of wild-type cells are in G_1.

Mutations Are Clustered Mainly in the FAT and Kinase Domains of Tor2p. The above analysis identified single amino acid changes located in the FAT and kinase domains, pointing to the importance of these two domains. To further investigate the significance of these domains for Tor2p activation, we screened for additional mutations in the C-terminal half of Tor2p. The region containing either the FAT or the FRB, kinase, and FATC domains of the *tor2* gene in JUp1050 was randomly mutagenized, and 34 additional mutants that exhibited RIG were identified. Sequence analysis of these mutants revealed 17 additional single mutations at 15 positions.

Fig. 2*A* summarizes all of the activating mutations identified. Interestingly, we found that the mutations mainly were clustered in two regions: the N-terminal side of the FAT domain (cluster I) and the C-terminal portion of the kinase domain (cluster II). In addition, there were a few regions, notably at the C-terminal region of the FAT domain and the N-terminal region of the kinase domain, where additional mutations were identified. Fig. 2*B* shows sequence alignments of residues in which mutations

Fig. 3. rhb1Δ tor2^{L1310F} and rhb1Δ tor2^{E2221K} show sensitivity to stresses. (*A*) JUp1050 (rhb1^{+} tor2^{+}), JUp1274 (rhb1^{+} tor2^{L1310F}), JUp1275 (rhb1Δ tor2^{L1310F}), JUp1261 (rhb1^{+} tor2^{E2221K}), and JUp1265 (rhb1Δ tor2^{E2221K}) were streaked on the indicated plates and incubated at the indicated temperatures. (*B*) JUp1265 (rhb1Δ tor2^{E2221K}) and JUp1275 (rhb1Δ tor2^{L1310F}) were transformed with either pREP41 (rhb1Δ) or pRPL-5pRhb1 (rhb1^{+}), and transformants were streaked onto the indicated plates and incubated at 30°C.

were found in clusters I and II. As can be seen, most of the mutations occur on residues that are perfectly conserved among TOR proteins from different organisms.

rhb1Δ tor2^{rig} Mutants Are Sensitive to Stress Conditions. Analysis of a strain having a disruption of *rhb1* and carrying a *tor2*-activated (*tor2^{rig}*) mutation revealed that, although Tor2 mutants can bypass Rhb1p requirement for growth, they are incapable of bypassing other Rhb1p functions. Fig. 3*A* shows that the *rhb1*Δ strains that are viable because of the presence of either the *tor2^{L1310F}* or the *tor2^{E2221K}* mutation are sensitive to high-salt stress (1 M KCl) and high temperature (37°C). This sensitivity can be reversed by the introduction of *rhb1*$^{+}$, indicating that Rhb1p is involved in responding to these stresses in fission yeast.

We previously have shown that inhibition of Rhb1p causes hypersensitivities to toxic analogues of lysine (thialysine) and arginine (canavanine) (25, 46). In addition, loss of *tsc2* results in resistances to thialysine, canavanine, and ethionine (the toxic analog of methionine) (22, 23, 25). Examination of the *rhb1*Δ *tor2^{L1310F}* and *rhb1* Δ *tor2^{E2221K}* mutants on these toxic amino acid analogs showed that these double mutants are hypersensitive to thialysine, canavanine, and ethionine (Fig. 3*B* and data not shown). These sensitivities are reversed by reintroducing *rhb1*$^{+}$ into these cells. Thus, Rhb1p is required for the resistance to these amino acid analogs, and, because these cells carry activated Tor2p, this resistance likely is independent of Tor2p.

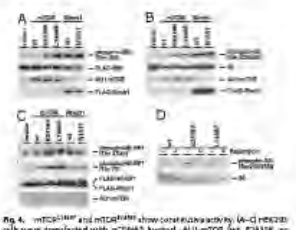

Fig. 4. mTOR^{L1460P} and mTOR^{E2419K} show constitutive activity. (*A–C*) HEK293 cells were transfected with pCDNA3 (vector), AU1-mTOR (wt, E2419K, or L1460P), or FLAG-Rheb1 (wt or N153T) to detect the phosphorylation of S6K or 4E-BP1. FLAG-S6K or FLAG-4E-BP1 was cotransfected. Cells then were serum-starved overnight and cultured in FBS for 1 h. The protein levels were detected by anti-FLAG (anti-S6K, 4E-BP1, and Rheb), anti-AU1 (mTOR), or anti-S6 antibody. The phosphorylation levels of S6K, 4E-BP1, and S6 were detected by phospho-specific antibodies. (*D*) HEK293 cells were transfected with AU1-mTOR (wt, E2419K, or L1460P). Cells were serum-starved overnight and then treated with rapamycin (100 nM). The expression and phosphorylation levels of S6 were detected by specific antibodies. wt, wild type.

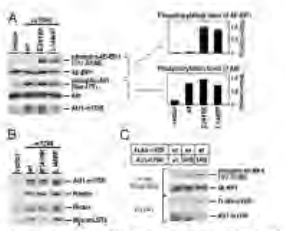

Fig. 5. Kinase activities of mTOR complexes. (*A*) HEK293 cells transfected with pCDNA3 (vector) or AU1-mTOR (wt, E2419K, or L1460P) were serum-starved overnight and then cultured in FBS for 1 h. AU1-mTOR complexes then were immunoprecipitated with anti-AU1 antibody and used for in vitro kinase assays with 4E-BP1 and Akt as substrates. Phosphorylation of substrates was detected by use of the indicated phospho-specific antibodies. Levels of phosphorylation of substrates were quantitated relative to that by mTOR^{L1460P} and graphed. (*B*) HEK293 cells were transfected with pCDNA3 (vector) or AU1-mTOR (wt, E2419K, or L1460P) together with Myc-mLST8. Cells were serum-starved overnight and cultured for 1 h in FBS. AU1-mTOR complexes then were immunoprecipitated with anti-AU1 antibody. mTOR were detected by anti-AU1 antibody. mLST8 were detected by anti-Myc antibody. Raptor and Rictor were detected by specific antibodies. (*C*) HEK293 cells cotransfected with FLAG-mTOR and AU1-mTOR (wt, E2419K, or L1460P) were serum-starved overnight and cultured for 1 h in FBS. mTOR dimers were immunoprecipitated by using anti-FLAG antibody and used for in vitro kinase assays with 4E-BP1 as substrate. FLAG-mTOR and AU1-mTOR were detected by anti-FLAG and anti-AU1 antibodies, respectively. Phosphorylation of substrate was detected by use of anti-phospho-4E-BP1 (Thr 37/46).

Analogous Mutations in mTOR Confer Nutrient-Independent Activity. Because the mutations we identified occur mostly on residues that are conserved in higher eukaryotes, we asked whether these mutations would confer constitutive activation of mTOR. To examine this point, mTOR^{L1460P} and mTOR^{E2419K}, mutations that correspond to Tor2^{L1943P} and Tor2^{E2202K}, respectively, were constructed, and the regulation of their activities by nutrient signals in HEK293 cells was assessed. Consistent with previous reports (47, 48), mTOR activity as detected by the phosphorylation of S6K, S6, or 4E-BP1 is inhibited when the cells are exposed to nutrient-starvation conditions (Fig. 4 *A–C*). This inhibition is overcome by overexpressing wild-type Rheb or a Rheb^{N153T} mutant, which is analogous to the hyperactive fission yeast Rhb1^{N153T} mutant that we previously have shown to be highly bound to GTP (Fig. 4 *A–C*) (25, 49). The activation seen by wild-type Rheb is likely attributable to Rheb being highly bound to GTP when transiently expressed (50).

We then examined the mTOR mutants. HEK293 cells were transfected with constructs that expressed either wild-type or the mutant mTOR (L1460P or E2419K) and then starved for nutrients. As can be seen in Fig. 4 *A*, *B*, and *C*, cells expressing mTOR^{L1460P} and mTOR^{E2419K} exhibit high phosphorylation of S6K, 4E-BP1, and S6 compared with wild-type mTOR, indicating that the mTOR mutants maintain activity even when the cells are starved for nutrients. However, these mTOR mutants retain sensitivity to inhibition by rapamycin (Fig. 4*D*). mTOR activity (S6 phosphorylation) was assessed in HEK293 cells expressing wild-type mTOR, mTOR^{L1460P}, or mTOR^{E2419K} and treated with either DMSO (−) or rapamycin (+). We found that both mTOR mutants were sensitive to rapamycin, similar to wild-type mTOR.

Constitutive activation of the mTOR mutants also can be examined by measuring *in vitro* kinase activities. The activities of the two mTORCs were assayed by using two different substrate proteins: mTORC1 activity was assayed by using 4E-BP1 (51, 52), whereas mTORC2 was assayed by using Akt as a substrate protein (53, 54). These complexes were immunoprecipitated from nutrient-starved cells by using AU1-tagged wild-type or mutant mTOR and activities assessed *in vitro* (Fig. 5*A*). Activity using Akt as the substrate was still retained with wild-type mTOR in HEK293 cells even under nutrient-starved conditions, and no change in this activity was observed when using the mutant mTOR. On the other hand, we found that the mTOR complex containing mTOR^{L1460P} or mTOR^{E2419K} exhibited significantly higher activity with 4E-BP1 as the substrate relative to wild-type mTOR. This activity was confirmed to be attributable to mTORC1, because an *in vitro* kinase assay using mTORC1 immunoprecipitated with anti-Raptor antibody showed similar results (SI Fig. 7). These results are consistent with our *in vivo* findings.

mTOR^{L1460P} and mTOR^{E2419K} Mutants Can Form mTORC1 and mTORC2 Complexes and an Active Heterodimer with Wild-Type mTOR. We asked whether there were any alterations in the ability of the mTOR mutants to form mTORC1 and mTORC2. By using the AU1 tag on the expressed mTOR, mTOR complexes were immunoprecipitated from HEK293 cells under nutrient-starvation conditions, and levels of Raptor (mTORC1), Rictor (mTORC2), and mLST8 (mTORC1 and mTORC2) were assessed (Fig. 5*B*). We found that the mTOR mutants were able to bind similar amounts of these mTORC1 and mTORC2 components as was wild-type mTOR.

It has been reported that mTOR dimerizes via its N-terminal HEAT domains and that the dimeric mTORC1 is the major form that responds to insulin (36, 38). Because our mutations are not located in the HEAT domains, it is likely that wild-type and mutant mTOR would form a heterodimeric complex. To test whether this heterodimer exhibits constitutive activation of mTOR function, we coexpressed FLAG-tagged wild-type and AU1-tagged wild-type or mutant mTOR proteins in HEK293 cells. The cells were starved for nutrients, and the complexes were isolated by immunoprecipitation using anti-FLAG antibody. We found similar amounts of AU1-tagged wild-type mTOR and the AU1-tagged mutant mTOR in the immune complex, indicating that mutant mTOR was able to form a heterodimer with wild-type mTOR (Fig. 5*C*). Furthermore, when these complexes were assayed for activity by using 4E-BP1 as a substrate, we found that, although the wild-type/wild-type dimers were inactive, the wild-type/mutant heterodimers exhibited *in vitro* kinase activity (Fig. 5*C*).

Discussion

Accumulating evidence in fission yeast provides strong support for the idea that Rhb1p is an activator of Tor2p. We have reported that Rhb1p associates with Tor2p (25). We and others also recently have shown that, in fission yeast, Tor2p complexes with Mip1p (the fission yeast Raptor homolog), likely forming a SpTORC1 (44, 45). Furthermore, shutting down Tor2p results in small rounded cells arrested in G_1, reminiscent of inhibiting Rhb1p (44, 45). In addition, inhibition of Tor2p in homothallic cells induces mating and sexual development (44, 45). Now, we show that, opposite to inhibiting Tor2p function, an activating mutation in Tor2p can confer R1G, delayed response to nitrogen starvation, and decreased sexual development.

This report provides evidence for a large number of single amino acid changes that confer constitutive activation of Tor2p. Importantly, many of these mutations occur in residues that are highly conserved in TOR proteins, and mTOR carrying analogous mutations exhibit nutrient-independent mTORC1 activation. An exciting finding of our study is that the activating mutations are clustered within two domains of TOR: the N-terminal region of the FAT domain (cluster I) and the C-terminal region of the kinase domain (cluster II). Interestingly, the cluster in the kinase domain is just adjacent to the repressor domain (2430–2450 of mTOR; Fig. 2*B*); deletion of this region results in activation of mTOR (39, 40). Indeed, the analogous position (2431 of mTOR) for one of the activated fission yeast Tor2 mutants, L2233H, is found just inside this repressor domain. This close proximity of the repressor domain to cluster II possibly indicates that these mutations may activate mTOR via a similar mechanism. The deletion of the repressor domain initially was thought to activate mTOR, in part by removing an Akt phosphorylation site (S2448; Fig. 2*B*); however, mutating this site, which later was shown to be an S6K phosphorylation site (42, 43), to alanine does not significantly alter mTOR activity (39). Furthermore, mutating the AMPK phosphorylation site (T2446; Fig. 2*B*) to alanine did not alter mTOR activity (39, 41). These phosphorylation sites also are not conserved in fission yeast Tor2p. Perhaps this region is involved in interacting with an unknown inhibitory factor. In addition to cluster II, mutations were found in the N-terminal region of the kinase domain. Interestingly, Rheb is reported to interact with the N-terminal half of the mTOR kinase domain (29). It is possible that these mutations in the kinase domain mimic the effects of Rheb binding, which may interfere with this inhibitory factor. Further experiments are needed to address the consequences of these mutations. A recent report in budding yeast identified mutations in the FRB that exhibit increased association with KOG1p (budding yeast Raptor homolog) and increased activity to *Saccharomyces cerevisiae* TOR1p (55). Although the FRB region was included in our mutagenesis, we did not identify any activating mutation in this region.

The activation of Tor2p results in a delay in the nitrogen-starvation response. However, this response is not a complete block. Furthermore, we find that *rhb1*Δ *tor2*^{ts3} strains also are able to respond to nitrogen starvation and undergo sexual differentiation (SI Fig. 8). The observation that these strains still are able to respond to nitrogen starvation in the absence of Rhb1p raises the possibility that Tor2p also is regulated by an Rhb1p-independent mechanism. Further analysis of *rhb1*Δ *tor2*^{ts3} cells also revealed possible involvement of Rhb1p in stress response. These mutants are sensitive to stresses such as high salt (1 M KCl) and high temperatures (37°C). Although the mechanisms for these phenotypes need further investigation, it is of interest to note that *tor1*Δ mutants also exhibit these phenotypes. Another phenotype of the *rhb1*Δ *tor2*^{ts3} mutants is that they are hypersensitive to toxic amino acids analogs. It is possible that these sensitivities are a result of increased amino acid uptake because we previously have shown that decreases in Rhb1p function lead to increased uptake of arginine and hypersensitivity to canavanine (46).

We have succeeded in identifying mTOR mutants that are active independent of nutrients. These mutants provide valuable reagents to further examine the biological consequences of mTOR activation. By introducing these mutants in HEK293 cells, we have shown that they confer nutrient-independent mTORC1 signaling. Additional experiments may shed light onto the role of mTOR activation in growth. Introducing these mutants in HEK293 as well as other untransformed cell lines also may provide insight into mTOR activation in transformation. Investigating mTOR activation in whole animals also is of importance. These mutants can be introduced into mice, and such animals can be studied further for roles of mTOR in development as well as propensity for tumors.

Activation of the mTOR pathway has been implicated in a number of human diseases associated with benign tumors, such as hamartomas (56). Our demonstration that mTOR can be activated by single amino acid changes raises an interesting possibility that mTOR mutations may be found in tumor samples. This idea is supported further by our finding that our mutant mTOR is active even when in a heterodimer with the wild-type protein. Because our study points to two hot spots in the TOR protein, searches for mTOR mutants may be focused on these two regions. The results obtained from these studies should have significant implication for our understanding of human diseases arising from the activation of the TSC/Rheb/mTOR signaling pathway.

Materials and Methods

Screens for RIGs and Activated Tor2p. *RIG screen.* The scheme for the RIG screen is shown in Fig. 1. JUp1050 strain (3×10^8 cells) was mutagenized with methyl-nitro-nitrosoguanidine (Sigma, St. Louis, MO) and recovered in rich yeast extract with supplements (YES) media overnight. Cells then were plated onto Edinburgh minimal medium supplemented with adenine (225 mg/l), leucine (225 mg/l), uracil (50 mg/l), and FOA (1 g/l). Plates were replica-plated once to eliminate background. Of ≈3,000 colonies, 241 clones were isolated and tested for absence of *rhb1*$^+$ by colony PCR. One clone was isolated that had lost the *rhb1*$^+$ plasmid.

Screen for tor2 mutants exhibiting decreased mating. Random mutations were introduced into the *tor2* gene by PCR (57). Linear DNA fragments carrying mutagenized *tor2* alleles were transformed into JV530, a homothallic strain in which the endogenous *tor2* gene is disrupted with a kan^r cassette and whose growth is maintained by a multicopy plasmid pREP42-tor2. To obtain integrants of functional *tor2* alleles, transformants that were resistant to FOA (indicating loss of *ura4*$^+$-based pREP42-tor2) and kan^r were screened at 26.5°C. From a *tor2* mutant library thus constructed, we screened for sterile clones; each strain was grown to a colony on SSA plate (see *SI Materials and Methods*) and stained with iodine vapor after incubation for 4 days at 30°C. Unstained colonies were isolated and examined microscopically for sterility.

Screen for additional FAT and kinase mutants. Additional FAT and kinase domain mutations were identified by screening libraries based on pUG6-tor2-CTL in which the region containing the FAT domain or the FRB, kinase, and FATC domains were randomly mutagenized by using the GeneMorph II Random Mutagenesis Kit (Stratagene, La Jolla, CA). The libraries were digested with BamHI, and the linearized plasmids were integrated into JUp1050. The resulting transformants initially were selected on plates containing G418 (200 μg/l) for integration of plasmid and then on FOA for the RIG phenotype as before. Then, 8,400 and 12,000 integrants were screened from the FAT domain library and the FRB-kinase-FATC domain library, respectively. In both cases, 17 clones were isolated. The regions that were mutagenized were PCR-amplified from genomic preps and sequenced to identify the mutations.

Mammalian Cell Culture and Transfection. HEK293 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C and 5% CO_2. Transfections were performed by using Polyfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. To assess the activity of mTOR mutants, cells were serum-starved in DMEM supplemented with 0.1% BSA overnight and then cultured in PBS for 1 h. For rapamycin treatment, cells were treated with 100 nM rapamycin for 1 h after serum starvation. These cells were lysed, and proteins were analyzed by Western blotting analysis.

Immunoprecipitation and *In Vitro* Kinase Assay. Cells were lysed with buffer A [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% CHAPS, 1 mM $MgCl_2$ and 1 mM EDTA]. The supernatant from the centrifugation at 20,000 × *g* for 15 min was incubated with anti-AU1 antibody (Covance, Berkeley, CA) and protein G-Sepharose 4FF beads (Amersham Biosciences, Piscataway, NJ) at 4°C for 2 h. Immunoprecipitates were washed three times with buffer A. For *in vitro* kinase assay, immunoprecipitates were incubated in kinase buffer [100 mM Tris·HCl (pH 7.5), 50 mM $MgCl_2$, and 1 mM ATP] containing 0.5 μg GST-4E-BP1 or Akt for 30 min at 37°C. Samples were boiled in SDS sample buffer [3% SDS, 5% glycerol, 62 mM Tris·HCl (pH 6.7)], and proteins were analyzed by Western blotting analysis.

SI. Additional information regarding yeast strains, media and manipulations, cell cycle and size analysis, plasmid constructs, and antibodies and reagents is provided as *SI Materials and Methods*. A list of strains used in this study is provided in SI Table 1.

We thank the University of California, Los Angeles, Flow Cytometry Core facility and Lea Gao for assistance with FACS analysis. This work was supported by National Institutes of Health Grant CA41936 and Department of Defense Grant W81XWH-05-1-0264 (to F.T.) and by a Grant-in-Aid for Specially Promoted Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan (to M.Y.).

1. Aagaard PJ, Tamanoi F (2004) *Cell Signal* 16:1105–1112.
2. Avruch J, Lin Y, Long X, Murthy S, Ortiz-Vega S (2005) *Curr Opin Clin Nutr Metab Care* 8:67–72.
3. Dann SG, Thomas G (2006) *FEBS Lett* 580:2821–2829.
4. Inoki K, Guan KL (2006) *Trends Cell Biol* 16:206–212.
5. Wullschleger S, Loewith R, Hall MN (2006) *Cell* 124:471–484.
6. Astrinidis A, Henske EP (2005) *Oncogene* 24:7475–7481.
7. Tee AR, Blenis J (2005) *Semin Cell Dev Biol* 16:29–37.
8. Ikeo K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K (2002) *Cell* 110:177–189.
9. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2002) *Cell* 110:163–175.
10. Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM (2003) *Mol Cell* 11:895–904.
11. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN (2004) *Nat Cell Biol* 6:1122–1128.
12. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2004) *Curr Biol* 14:1296–1302.
13. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B (2006) *Cell* 127:125–137.
14. Pópulo MA, Thomsen CC, Jaffe JD, Schreiber W, Scolley T, Carr SA, Sabatini DM (2006) *Curr Biol* 16:1865–1870.
15. Yang Q, Inoki K, Ikenoue T, Guan KL (2006) *Genes Dev* 20:2820–2832.
16. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN (2002) *Mol Cell* 10:457–468.
17. Narayanan V (2003) *Pediatr Neurol* 29:404–409.
18. Gomez MR, Sampson JR, Whittemore VH (1999) *Tuberous Sclerosis Complex* (Oxford Univ Press, New York).
19. Mach KE, Furge KA, Albright CF (2000) *Genetics* 155:611–622.
20. Yang W, Tabancay AP, Jr, Urano J, Tamanoi F (2001) *Mol Microbiol* 41:1339–1347.
21. Matsumoto S, Bandyopadhyay A, Kwiatkowski DJ, Maizel U, Matsumoto T (2002) *Genetics* 161:1053–1063.
22. van Slegtenhorst M, Carr E, Stoyanova R, Kruger WD, Henske EP (2004) *J Biol Chem* 279:12706–12713.
23. van Slegtenhorst M, Mustafa A, Henske EP (2005) *Hum Mol Genet* 14:2851–2858.
24. Nakase Y, Fukada K, Chikashige Y, Tsutsumi C, Morita D, Kawamoto S, Ohnuki M, Hiraoka Y, Matsumoto T (2006) *Genetics* 172:569–578.
25. Urano J, Comiso MJ, Guo L, Aagaard PJ, Dunkhlin B, Tabancay AP, Jr, Kato-Stankiewicz J, Tamanoi F (2005) *Mol Microbiol* 58:1074–1086.
26. Kawai M, Nakashima A, Ueno M, Ushikaru T, Aiba K, Doi H, Uritani M (2001) *Curr Genet* 39:166–174.
27. Weisman R, Choder M (2001) *J Biol Chem* 276:7027–7032.
28. Weisman R, Roitburg I, Nahari T, Kupiec M (2005) *Genetics* 169:539–550.
29. Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J (2005) *Curr Biol* 15:702–713.
30. Long X, Ortiz-Vega S, Lin Y, Avruch J (2005) *J Biol Chem* 280:23433–23436.
31. Smith EM, Finn SG, Tee AR, Browne GJ, Proud CG (2005) *J Biol Chem* 280:18717–18727.
32. Kunz J, Schneider U, Howald I, Schmidt A, Hall MN (2000) *J Biol Chem* 275:37011–37020.
33. Sabatini DM, Barrow RK, Blackshaw S, Burnett PE, Lai MM, Field ME, Bahr BA, Kirsch J, Betz H, Snyder SH (1999) *Science* 284:1161–1164.
34. Wu S, Mikhailov A, Kalo-Hennis H, Ikeo K, Yonezawa K, Avruch J (2002) *Biochim Biophys Acta* 1542:41–56.
35. Choi JH, Bertram PG, Drenan R, Carvalho J, Zhou HH, Zheng XF (2002) *EMBO Rep* 3:988–994.
36. Takahara T, Ikeo K, Yonezawa K, Sorimachi H, Maeda T (2006) *J Biol Chem* 281:28605–28614.
37. Wullschleger S, Loewith R, Oppliger W, Hall MN (2005) *J Biol Chem* 280:30697–30704.
38. Wang L, Rhodes CJ, Lawrence JC, Jr (2006) *J Biol Chem* 281:24293–24303.
39. Sekulic A, Hudson CC, Homme JL, Yin P, Otterness DM, Karnitz LM, Abraham RT (2000) *Cancer Res* 60:3504–3513.
40. Edinger AL, Thompson CB (2004) *Oncogene* 23:5654–5663.
41. Cheng SW, Fryer LG, Carling D, Shepherd PR (2004) *J Biol Chem* 279:15719–15722.
42. Chiang GG, Abraham RT (2005) *J Biol Chem* 280:25485–25490.
43. Hidir MK, Blenis J (2005) *J Biol Chem* 280:26089–26093.
44. Alvarez B, Moreno S (2006) *J Cell Sci* 119:4475–4485.
45. Matsuo T, Otsubo Y, Urano J, Tamanoi F, Yamamoto M (2007) *Mol Cell Biol*, in press.
46. Yang W, Urano J, Tamanoi F (2003) *J Biol Chem* 278:429–438.
47. Wang X, Campbell LE, Miller CM, Proud CG (1998) *Nucleic Acids Res* 26:2675–2685.
48. Ikeo K, Yonezawa K, Wang QP, Kodowaki MT, Balhan C, Avruch J (1998) *J Biol Chem* 273:14484–14494.
49. Yan L, Findlay GM, Jones R, Procter J, Cao Y, Lamb RF (2006) *J Biol Chem* 281:19793–19797.
50. Im E, von Lintig FC, Chen J, Zhuang S, Qui W, Chowdhury S, Worley PF, Boss GR, Pike LB (2002) *Oncogene* 21:6356–6365.
51. Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, Lawrence JC, Jr, Abraham RT (1997) *Science* 277:99–101.
52. Brunn GJ, Fadden P, Haystead TA, Lawrence JC, Jr (1997) *J Biol Chem* 272:32547–32550.
53. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) *Science* 307:1098–1101.
54. Hresko RC, Mueckler M (2005) *J Biol Chem* 280:40406–40416.
55. Nojima A, Chen IC, Aronova S, Powers T (2006) *J Biol Chem* 281:31616–31626.
56. Inoki K, Corradetti MN, Guan KL (2005) *Nat Genet* 37:19–24.
57. Zhou YH, Zhang XP, Ebbright RH (1994) *Nucleic Acids Res* 19:6052.

Appendix 2

Proceedings American Association for Cancer Research, Volume 46, 2005
Abstract #5434
Cellular and Molecular Biology 67: Cell Cycle Control and Cancer 3

The regulation of cell cycle progression by Tsc and Rheb GTPase

Susie M. Miyamoto, Juran Kato-Stankiewicz, Chen Jiang, Chia-Ling Gau, Lea Guo and Fuyuhiko Tamanoi

UCLA, Los Angeles, CA

Tsc and Rheb are two important players of the PI3K/AKT/TSC/mTOR signaling pathway that has been shown to regulate cell cycle progression in addition to other cellular processes including proliferation, tumorigenesis, angiogenesis, differentiation, and anti-apoptosis. Tsc1 and Tsc2 form a complex that functions as a GTPase activating protein (GAP) for Rheb. Mutations in the Tsc1 or Tsc2 genes have been implicated in tuberous sclerosis, a genetic disorder marked by the appearance of benign tumors called hamartomas in multiple organs. Previously we used *Tsc2*$^{-/-}$ MEFs as a model to investigate the function of the TSC/Rheb/mTOR pathway. We have shown that *Tsc2*$^{-/-}$ MEFs escape cell cycle arrest in G0/G1 at high confluency and in serum-starved conditions (Gau *et al*., AACR meeting, 2004). However, the mechanism of how TSC/Rheb/mTOR induces cell cycle progression is not known. Our analysis of *in vitro* kinase assays revealed that CDK2 kinase activity is significantly increased in *Tsc2*-null MEFs. We have also examined expression levels of various cyclins, CDKs, and CDK inhibitors. No significant differences in the level of expression of CDK2, p27, and p21 were detected between *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. However, we did observe a difference in the cellular localization of p27 between the two cells. Biochemical fractionation experiments showed that p27 is localized in both cytoplasmic and nuclear fractions in *Tsc2*$^{+/+}$ MEFs. In contrast, p27 was localized only in the cytoplasmic fraction in *Tsc2*$^{-/-}$ MEFs. There is no change in the localization of p21. This lack of nuclear localization of p27 may explain the increased activity of CDK2 in *Tsc2*$^{-/-}$ MEFs. It is interesting to point out that cytoplasmic translocation of p27 has been detected in a number of cancer cells. In addition, cytoplasmic translocation of p27 has been detected in breast cancer and HEK293T cells upon Akt activation. Furthermore, TSC2 was found to bind p27 to protect it from proteasomal degradation. Rheb is clearly implicated in cell cycle progression, as overexpression of Rheb in HEK293 cells promoted an S phase progression. Since the Tsc1/Tsc2 complex negatively regulates Rheb, a question arises regarding the role of Rheb in cell cycle progression. Further analysis of how Rheb regulates G1/S cell cycle associated proteins will be discussed. These results provide insight into how Tsc and Rheb are involved in the regulation of cell cycle progression. This also implicates the possibility of taking advantage of drugs such as farnesyltransferase inhibitors that target Rheb to influence cell cycle progression.