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Award Number: W81XWH-09-1-0279

TITLE: Regulation of mTOR by Nutrients

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REPORT DATE: July 2010

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE July 2010		2. REPORT TYPE Annual		3. DATES COVERED 1 July 2009 – 30 June 2010	
4. TITLE AND SUBTITLE Regulation of mTOR by Nutrients				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0279	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kun-Liang Guan E-Mail: kuguan@ucsd.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of San Diego La Jolla, CA 92093				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 The major functional of the TSC1 and TSC2 tumor suppressor genes is to inhibit the activity of the mammalian target of rapamycin (mTOR), which is a key cell growth regulator. mTOR forms two distinct physical and functional complexes, TORC1 and TORC2. Constitutive TORC1 activation contributes to tuberous sclerosis development. TORC1 is regulated by a wide range signals, including growth factors, cell energy levels, and nutrients. Amino acids are the most potent TORC1 activators, however the molecular mechanism of amino acid signaling to TORC1 activation is largely unknown. Recent works from our laboratory have identified the Rag GTPases in relaying amino acid signals to mTOR. We have showed the importance of Rag GTPases in TORC1 regulation and its relationship with the Rheb GTPase. Moreover, we have generated data support the Rab family GTPases in TORC1 regulation. These data provide new insights into the mechanism of TORC1 activation by amino acid signals.					
15. SUBJECT TERMS Tuberous sclerosis, TSC1/2, mTOR, Rag, Rheb					
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
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Table of Contents

	<u>Page</u>
Introduction.....	2
Body.....	2
Key Research Accomplishments.....	4
Reportable Outcomes.....	5
Conclusion.....	5
References.....	5
Appendices.....	6

Introduction

Previous studies have established that activation of the mammalian target of rapamycin complex 1 (TORC1) is the major pathophysiological consequence in cells with mutation of either TSC1 or TSC2, the two tumor suppressor genes that mutations are responsible for the development of tuberous sclerosis complex, TSC. TORC1 is regulated by many upstream signals, including growth factors, cellular energy levels, and amino acid availability. The TSC1/TSC2 complex functions as a GTPase activating protein (GAP) to inhibit Rheb, which is a Ras family GTPase directly involved in TORC1 activation. We have shown that the Rag GTPases play a critical role in TORC1 activation in response to amino acid stimulation. This proposal aims to investigate the mechanism of the Rag GTPases in regulation of TORC1 activation.

Body

Amino acids are the most important activator of TORC1 (Wullschleger et al., 2006). In the absence of amino acids, neither growth factors nor glucose can effectively activate TORC1 (Hara et al., 1998). Studies from our laboratory identified Rag GTPases as key regulators of TORC1 in response to amino acids (Kim et al., 2008). Similar observations were also reported by David Sabatini's group (Sancak et al., 2008). These studies have established Rag GTPases as key signaling molecules acting between amino acids and TORC1.

Rag GTPases are unique that they form a hetero dimer. RagA or RagB forms a hetero dimer with either RagC or RagD. It is the heterodimer that is functional in TORC1 activation. However, overexpression of the RagA or RagB can activate TORC1 though less efficiently. In contrast, overexpression of either RagC or RagD cannot activate TORC1. These results suggest that the two Rag subunits in the heterodimer do not functional equally. The N-terminal region of Rag contains the GTP binding and GTPase domain. To further support the importance of Rag dimer in TORC1 activation, we have mapped that the C-terminal regions of both RagA/B and RagC/D are important for dimer formation. Without the C-terminal region, the truncated Rag GTPases are not functional in TORC1 activation. These data demonstrate that both the N-terminal GTPase domain and the C-terminal dimerization domain are important for Rag function in vivo.

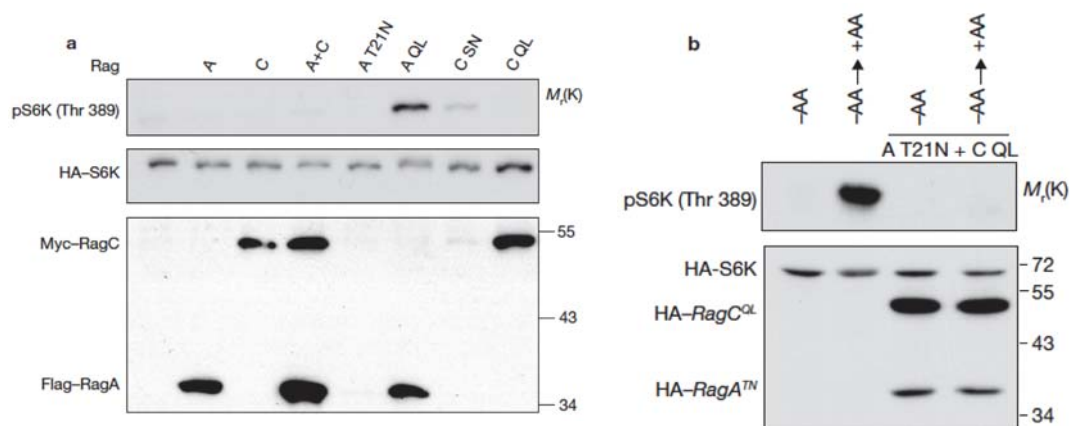


Fig.1. Rag GTPases are involved in TORC1 activation in response to amino acids. (a) Constitutively active RagA and RagB stimulate S6K phosphorylation. Mammalian RagA, RagB, RagC, or RagD construct was co-transfected with HA-S6K into HEK293 cells. Their

corresponding dominant negative mutants (RagA T21N, RagB T54N, RagC S75N, RagD S76N), and constitutively active mutants (RagA Q66L, RagB Q99L, RagC Q120L, RagD Q121L), were also tested. Phosphorylation and protein levels were determined by immunoblotting with appropriate antibodies, as indicated. Expression of RagA Q66L and RagC S75N (to a less degree) activated TORC1 in the absence of amino acids as indicated by the increased S6K phosphorylation. **(b)** RagA has a dominant role over RagC in regulating S6K phosphorylation. Each indicated Rag constructs were co-transfected with HA-S6K. The different Rag mutants used in the transfection are indicated on the top of each lane. The presence or absence of amino acids (AA) is also indicated. Phosphorylation of S6K was determined.

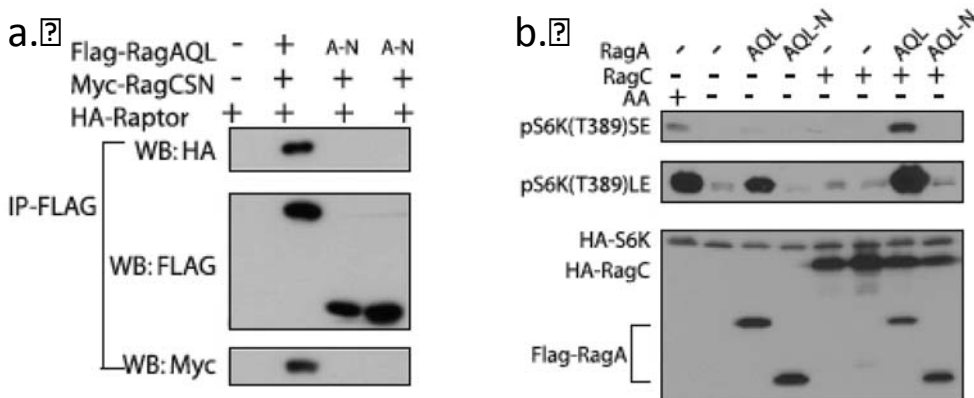


Fig.2. Dimerization is required for RagA/C to activate TORC1. **(a)** The C-terminal domain is required for RagA to interact with raptor. Flag-RagA full length or N-terminal domain (A-N) was co-transfected with Myc-RagC and HA-Raptor. Cell lysates were precipitated with Flag antibody and the co-immunoprecipitated HA-Raptor was detected by Western blot (top panel). **(b)** The C-terminal domain is required for RagA to stimulate S6K phosphorylation. HA-S6K was co-transfected with full length or N-terminal domain of RagA-QL in the presence or absence of RagC as indicated. The transfected cells were treated with medium with or without amino acids (AA) as indicated. Phosphorylation of S6K was determined to indirectly measure TORC1 activity. SE and LE denote for short exposure and long exposure, respectively.

We investigated the relationship between Rag and Rheb, which is also a Ras family GTPase. Rheb is inactivated by the TSC1/TSC2 GAP activity (Li et al., 2004). Rheb can directly activate TORC1. Dominant negative RagA mutant can block amino acid-induced but not Rheb-induced TORC1 activation, indicating that Rag does not function downstream of Rheb. Given the fact that Rheb can directly bind to and activate TORC1 and RagA can potentially bind Raptor, a key subunit of TORC1, our data suggest a model that Rag functions parallel to the TSC-Rheb signaling branch.

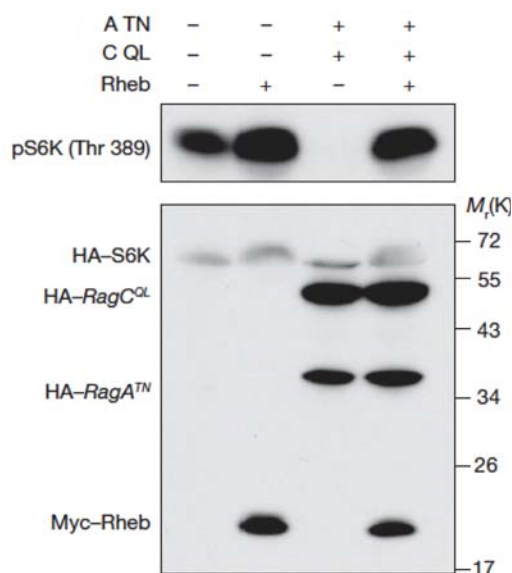


Fig.3. Rag GTPases act parallel to Rheb in TORC1 activation. Dominant negative RagA T21N and RagC Q120L were transfected into HEK293 cells with or without Rheb construct. S6K was included in the co-transfection. Phosphorylation and protein levels of the transfected proteins were determined by immunoblotting with appropriate antibodies, as indicated. Dominant negative RagA does not inhibit TORC1 activation by Rheb.

We performed functional screen to search for novel TORC1 regulators, especially in response to amino acid stimulation. Our RNA interference screen using *Drosophila* S2 cells has also isolated Rab and Arf as potential regulators of TORC1 (Li et al.). Both Rab and Arf are Ras family GTPases and have been implicated in intracellular trafficking (Gillingham and Munro, 2007; Zerial and McBride, 2001). Uncontrolled Rab5 and Arf1 potentially inhibit TORC1. This inhibitory effect is specific to amino acid stimulation but does not interfere TORC1 activation by glucose. These observations demonstrate a critical role of intracellular trafficking in amino acid induced TRC1 activation. Our data are consistent with a recent report that amino acid stimulation promotes TORC1 recruitment to lysosomal membrane where TORC1 is activated by Rheb (Sancak et al.).

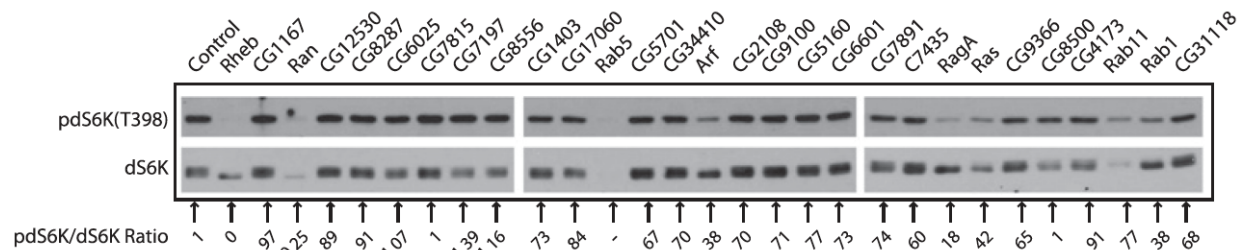


Fig.4. Rab and Arf proteins are involved in regulating TORC1 activity in *Drosophila* S2 cells. *Drosophila* S2 cells untreated (lane 1) or treated with the double stranded RNA against individual genes (as indicated by the *Drosophila* genome CG numbers) were starved of amino acids for 1 h followed by amino acid stimulation for 30 min. Phosphorylation and protein levels of dS6K were determined by immunoblotting with the indicated antibodies. Signals detected by anti-pdS6K and anti-dS6K were quantified and the ratio was calculated. An example of the screen results is shown. The control ratio is set to be 1 and all other ratio is the comparison with the control.

To further investigate the mechanism of Rag in TORC1 regulation, we explored the three dimensional structure in collaboration with Dr. Yanhui Xu. Initial efforts on crystallization of RagA/RagC complex was not successful. We then focused our attention on Gtr1/Gtr2 complex, which are the yeast homologs of the mammalian Rag GTPases. To this end, we have obtained crystal of Gtr1/Gtr2 complex and are in the process of solving the three dimensional structure of the Gtr1/Gtr2 complex.

Key Research Accomplishments

The research accomplishments in the last year are

1. We have identified the switch I as the effector domain important for Raptor binding and TORC1 activation.
2. We have mapped the C-terminal region of RagA and RagC as important for GTPase dimerization and TORC1 activation.

3. We showed that amino acid-stimulated TORC1 activation is completely blocked by dominant negative RagA mutant, suggesting an essential role of Rag in amino acid signaling.
4. We observed that Rag acts parallel to TSC-Rheb in TORC regulation. In the absence of Rheb, Rag GTPases cannot activate TORC1.
5. We discovered that the Rab family GTPases are also involved in TORC1 regulation in response to amino acid stimulation, indicating a role of intracellular trafficking in amino acid-induced TORC1 activation.

Reportable Outcomes

Papers published on this project.

- Kim, E., Goraksha, P., Li, L., Neufeld, T.P., and Guan, K-L. (2008) Regulation of TORC1 by Rag GTPases in nutrient response. *Nature Cell Biol.* 10, 935-945.
- Kim, E. and Guan, K-L. (2009) RAG GTPases in Nutrient-mediated TOR Signaling Pathway. *Cell Cycle* 8, 1014-1018.
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- Li, L., Kim, E., Yuan, H., Inoki, K., Goraksha-Hicks, Schiesher, R. L., Neufeld, T.P., Guan, K-L., (2010) Regulation of mTORC1 by the Rab and ARF GTPases. *J. Biol. Chem.* 285, 19705-9.

Conclusion

During the past year, we have made significant progresses on this project. We have established that Rag GTPases as key signaling mediators between amino acids and TORC1. The Rag GTPases form a heterodimer to stimulate TORC1 activation by directly binding to raptor. Rag acts in a pathway parallel to TSC-Rheb upstream of TORC1. In addition, we have identified the Rab and Arf GTPases as important regulators of TORC1, especially in response to amino acid stimulation. These observations suggest an important role of intracellular trafficking in TORC1 activation by amino acids.

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Appendices