mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacular H⁺-ATPase

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The mTOR complex 1 (mTORC1) protein kinase is a master growth regulator that is stimulated by amino acids. Amino acids activate the Rag guanosine triphosphatases (GTPases), which promote the translocation of mTORC1 to the lysosomal surface, the site of mTORC1 activation. We found that the vacuolar H⁺-adenosine triphosphatase ATPase (v-ATPase) is necessary for amino acids to activate mTORC1. The v-ATPase engages in extensive amino acid–sensitive interactions with the Ragulator, a scaffolding complex that anchors the Rag GTPases to the lysosome. In a cell-free system, ATP hydrolysis by the v-ATPase is necessary for amino acids to regulate the v-ATPase-Ragulator interaction and promote mTORC1 translocation. Results obtained in vitro and in human cells suggest that amino acid signaling begins within the lysosomal lumen. These results identify the v-ATPase as a component of the mTOR pathway and delineate a lysosome-associated machinery for amino acid sensing.

Amino acids are the building blocks of proteins and intermediates in lipid and adenosine triphosphate (ATP) synthesis. They also initiate a signaling cascade that leads to activation of the master growth regulator mTOR complex 1 (mTORC1). This multicomponent protein kinase integrates inputs from growth factors as well as nutrient and energy supplies to control many biosynthetic and catabolic processes (1). Most signals upstream of mTORC1 converge on TSC1-TSC2, a heterodimeric tumor suppressor that negatively regulates the Rheb guanosine triphosphatase (GTPase), which is an essential activator of mTORC1 protein kinase activity (2, 3). In contrast, amino acids signal to mTORC1 by promoting its binding to a distinct family of GTPases, the Rag GTPases (4, 5). The Rags form heterodimers consisting of RagA or RagB, which are highly similar to each other, bound to RagC or RagD, which are also highly related. In an amino acid–sensitive fashion, the Rag GTPases recruit mTORC1 to the surface of lysosomes, which also contain Rheb (5). The

References and Notes

Acknowledgments: This was supported by American Lebanese Syrian Associated Charities-St. Jude, NIH, and Howard Hughes Medical Institute (B.A.S.), grants from NIH and Millennium Pharmaceuticals (J.W.H.), and Damon Runyon Cancer Research Foundation (E.J.B.). We thank S. Gygi, I. Kurinov, C. Ralston, R. Cassell, P. Rodrigues, K. Kodali, V. Pagala, R. Schedeman, D. W. Miller, S. Bozeman, D. J. Miller, J. Bollinger, and C. Rock for assistance, reagents, and/or discussions. D.C.S., J.K.M., B.A.S., and St. Jude Children’s Research Hospital have applied for a patent on uses of Ubc12 N-terminal acetylation for inhibiting neddylation. Research Collaboratory for Structural Bioinformatics structural accession codes: 3TDI, 3TDU, 3TDZ. Author contributions: D.C.S., J.K.M., and E.J.B. designed, performed, and analyzed experiments; D.C.S. and B.A.S. wrote the manuscript, with all authors contributing; J.W.H. and B.A.S. advised and assisted on all aspects.

Supporting Online Material
www.sciencemag.org/cgi/content/full/science.1209307/DC1
Materials and Methods
Figs. S1 to S10

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trimeric Ragulator complex, which comprises the p18, p14, and MP1 proteins, anchors the Rag GTPases to the lysosome and, like the Rags, is necessary for mTORC1 activation by amino acids (6). Together, the Ragulator and Rag heterodimer form an amino acid–regulated docking site for mTORC1 on the lysosomal surface.

Amino acid signaling has been proposed to begin, alternatively, at the plasma membrane or inside the cell, but this key issue remains unsettled (7–10). The localization of the Rag GTPases on lysosomes, but not on other Rheb-containing endomembranes, suggests that this organelle has an important role in amino acid signaling to mTORC1. To determine whether lysosome-associated processes and proteins participate in the activation of mTORC1 by amino acids, we used RNA interference (RNAi) in Drosophila S2 cells to reduce the expression of a number of genes with roles in lysosomal biogenesis and function (table S1). Double-stranded RNAs (dsRNAs) targeting most of the genes did not affect the amino acid–induced phosphorylation of the ribosomal protein S6 kinase (dS6K) on T389, a readout of dTORC1 activity (table S1). In contrast, dsRNAs targeting vhaAC39, vha16, vha100-1, and vha100-2, all encoding components of the vacuolar H+-ATPase (v-ATPase) that do not have other known targets (SalA), decreased the size of S2 cells (Fig. 1C). Consistent with the results in Drosophila cells, lentiviral short-hairpin RNAs (shRNAs) targeting human ATP6V0c, the ortholog of Drosophila vha16, suppressed amino acid–induced phosphorylation of S6K1 in human embryonic kidney (HEK) 293T cells (Fig. 1D and fig. S1B). These results implicate the v-ATPase in the activation of mTORC1 by amino acids.

The v-ATPase consists of multicomponent V0 and V1 domains and operates through an incompletely understood mechanism in which each cycle of ATP hydrolysis by the V1 sector generates torque that rotates the membrane domain of V0, known as the rotor. In turn, this movement enables the transfer of protons into the lysosomal lumen, causing its acidification (11). The macrolides concanamycin A (ConA) and salicylihalamide A (SalA) are structurally diverse inhibitors of the v-ATPase that do not have other known targets (11–14). In 293T cells, both ConA and SalA inhibited amino acid–induced phosphorylation of S6K1 in a concentration-dependent manner (Fig. 1, A and B). The inhibition of S6K1 phosphorylation occurred after short (15- to 60-min) treatment times (Fig. S2A) and without concomitant alterations in lysosomal morphology (Fig. S2, B and C) or inhibition of Akt phosphorylation, a readout of growth factor signaling (Fig. 1, E and F).

The finding that the v-ATPase and its activity are necessary for mTORC1 activation by amino acids led us to consider potential roles for it in the pathway. One possibility is that the v-ATPase functions downstream of amino acids and is part of the amino acid–induced signaling pathway that culminates in mTORC1 activation. Another conceivable function is that the proton gradient generated by the v-ATPase is required for amino acids to be transported into the cellular compartment where an amino acid sensor is located. To bypass the transport function, we tested whether the v-ATPase is required for alcohol ester derivatives of amino acids to activate mTORC1. These esters freely diffuse across membranes and, within the cytoplasm and lysosomes, are hydrolyzed by esterases into native amino acids (15). A mixture of amino acid esters or leucine methyl ester activated mTORC1 with efficiencies comparable to those of their respective native amino acids (fig. S1, C and D). Moreover, ConA also inhibited the S6K1 phosphorylation induced by amino acid esters (Fig. 1G and fig. S1E). Consistent with these findings, SalA also suppressed mTORC1 activation induced by cycloheximide, which, by inhibiting translation, boosts concentrations of intracellular amino acids (7, 16) (Fig. 1H). Thus,
these results are consistent with the v-ATPase having a role downstream of intracellular amino acids in the initiation or propagation of the amino acid–induced signal to mTORC1.

A key event in amino acid signaling is the Rag GTPase–mediated translocation of mTORC1 to the surface of lysosomes (5, 6). In cells treated with ConA or SalA or depleted of ATP6V0c, mTOR failed to cluster onto lysosomes in response to amino acids and instead was found in a diffuse staining pattern (Fig. 2, A and B, and fig. S3, A to C). Unlike the Ragulator (6), the v-ATPase appears not to be necessary for anchoring the Rag GTPases to the lysosomal surface, because pharmacological or RNAi-mediated inhibition of the v-ATPase did not affect lysosomal localization of RagC (Fig. 2C and fig. S3, C and D). To test whether the v-ATPase might function upstream of the Rag GTPases, we used a RagB mutant that is constitutively active (RagBGTP) and renders mTORC1 signaling insensitive to amino acid starvation (4, 5). If the v-ATPase is required for the activation of the Rag GTPases, expression of RagBGTP should rescue the defects in mTOR lysosomal recruitment and S6K1 phosphorylation caused by ConA and SalA. Indeed, in cells stably expressing RagBGTP, the lysosomal localization of mTOR and the phosphorylation of S6K1 were insensitive not only to amino acid starvation but also to ConA and SalA treatment (Fig. 2, D and E, and fig. S3F).

Consistent with these results, in knockin mouse embryonic fibroblasts (MEFs) that express active RagA (RagAGTP) from the endogenous RagA locus, SalA (Fig. 2F) and ConA (fig. S3G) did not block the constitutive S6K1 phosphorylation caused by RagAGTP. Collectively, these results place the v-ATPase downstream of amino acids but upstream of the activation of the Rag GTPases; they also exclude its involvement in other regulatory inputs to mTORC1, such as controlling Rheb activity (6).

We tested whether a physical interaction exists between the v-ATPase and the Rags or Ragulator or both. Semi-quantitative mass spectrometric analyses of anti-FLAG immunoprecipitates prepared from 293T cells expressing FLAG-tagged Ragulator components (p18 or p14) or RagB revealed the presence of many subunits of the v-ATPase (Fig. 3A). Immunoblot assays with antibodies to endogenous V0 (c and d1) and V1 (A, B2, and D) subunits confirmed that Ragulator communoprecipitates with the V0 and V1 domains (Fig. 3, B and C), whereas the Rags communoprecipitate with V1 subunits only (fig S4, A and B). Although easily detected in immunoblot assays as communoprecipitating with Ragulator, the c subunit of V0 was not detected by mass spectrometry, probably because of its highly hydrophobic nature. The v-ATPase did not communoprecipitate with lysosomal (LAMP1) or cytoplasmic (Metap2) control proteins (Fig. 3, B and C). In vitro assays with purified recombinant proteins verified a direct interaction between the V0 component d1 and p18, but not p14, and between the V1 component D with p18 and, to a lesser degree, with p14 (Fig. 3D). No direct interactions were detected between the Rag GTPases and purified v-ATPase subunits (fig. S4C), which is consistent with the relatively low abundance of v-ATPase subunits.
in the RagB immunoprecipitates analyzed by mass spectrometry (Fig. 3A). Thus, in addition to scaffolding the Rag GTPases to the lysosomal surface, Ragulator provides a physical and functional link between the v-ATPase and the Rag GTPases. Consistent with amino acids acting upstream of the v-ATPase, amino acids regulated the interaction between the V1 domain of v-ATPase and Ragulator and Rag GTPases. Amino acid starvation and stimulation strengthened and weakened, respectively, the interaction (Fig. 3E and fig. S4D). In contrast, amino acids did not affect the binding of Ragulator with the V0 domain of the v-ATPase (Fig. 3E) or of the V1 and V0 subunits with each other [as does glucose starvation (17)].

**Fig. 3.** Interaction of the v-ATPase with the Ragulator-Rag GTPases. (A) Cartoon summarizing mass spectrometry analyses of immunoprecipitates from HEK-293T cells expressing FLAG-p18 (left), FLAG-p14 (center), and FLAG-RagB (right). v-ATPase subunits are color-coded according to their peptide representation (scale at the far right). (B) Binding of Ragulator to the V0 domain. HEK-293T cells stably expressing FLAG-tagged p18 and p14 were lysed and subjected to FLAG immunoprecipitation (IP) followed by immunoblotting for V0c and V0d1. FLAG-LAMP1 and FLAG-Metap2 served as negative controls. (C) Binding of Ragulator to the V1 domain. HEK-293T cells stably expressing FLAG-tagged p18, p14, LAMP1, and Metap2 were lysed and subjected to FLAG immunoprecipitation followed by immunoblotting for V1A, V1B2, and V1D. (D) (Top) In vitro binding assays in which purified FLAG-p18 and FLAG-p14 were incubated with recombinant V0d1 fused to glutathione S-transferase (HA-GST-V0d1), immobilized on glutathione agarose beads. Samples were subjected to immunoblotting for FLAG to detect bound Ragulator components. HA-GST-Rap2A served as a negative control. (Bottom) In vitro binding assays in which purified FLAG-p18 and FLAG-p14 were incubated with recombinant V1D fused to glutathione S-transferase (HA-GST-V1D). HA-GST-metap2 served as a negative control. (E) The Ragulator-V1 interaction, but not the Ragulator-V0 interaction, is regulated by amino acids. HEK-293T cells stably expressing FLAG-tagged p18, p14, and Metap2 were deprived of amino acids for 90 min or deprived and then stimulated with amino acids for 15 min. After lysis, samples were subjected to FLAG immunoprecipitation (IP) followed by immunoblotting for the indicated proteins. (F) SalA blocks regulation of the Ragulator-V1 interaction by amino acids. HEK-293T cells stably expressing FLAG-tagged p18 and p14 were deprived of amino acids for 90 min or deprived and then stimulated with amino acids for 15 min, in the presence of DMSO or 2 μM SalA. Samples were lysed, FLAG-immunoprecipitated, and immunoblotted for the indicated proteins. (G) Cartoon summarizing the Ragulator–v-ATPase interactions identified in (A) to (F). Orange denotes regulation by amino acids; blue indicates lack of regulation.
The v-ATPase is large and complex and has many functions that cannot easily be teased apart in live cells (II, 18–22). Thus, to better understand its role in amino acid signaling to mTORC1, we developed a cell-free system that recapitulates the amino acid–induced binding of mTORC1 to the Rag GTPases on the lysosomal surface [see methods in the supporting online material (SOM)]. We prepared a light organelle fraction from 293T cells expressing FLAG-RagB that had been deprived of amino acids. The organelles were briefly stimulated with amino acids or amino acid esters and then incubated with cytosol containing Myc-tagged Raptor (Fig. 4A and fig. S5A). In this system, amino acids, and especially amino acid esters, increased binding of Myc-Raptor to FLAG-RagB–containing vesicles but not to control vesicles (Fig. 4A and fig. S5B). As expected, in preparations containing the FLAG-RagB(T20D) mutant, the binding of Myc-Raptor was constitutively high and largely insensitive to amino acids (fig. S5C and S5D). We also prepared purified lysosomes by immunocollecting them from 293T cells (see methods in SOM) (fig. S6, A to C). Again, amino acid esters induced binding of Myc-tagged Raptor to isolated lysosomes (fig. S6D). Cytosol appeared to be dispensable, because highly purified, FLAG-tagged Raptor showed amino acid–induced binding to organelles containing GST-tagged Rag heterodimers (fig. S6E). Thus, lysosomes contain all the machinery required for sensing amino acids and activating the Rag GTPases.

In the in vitro system, the alcohol esters of amino acids were more effective than native amino acids in inducing the RagB-Raptor interaction (Fig. 4A). A possible reason for this is that amino acids must enter and accumulate in lysosomes to initiate signaling, and that the amino acid esters do so more easily in the in vitro preparation (15). To test the requirement for an intralysosomal accumulation, we used treatments that permeabilize the lysosomal membrane and thus allow amino acids to leak out. Treatment of the organelle preparation with Streptolysin O, which introduces nanometer-sized holes into the lysosomal membrane, or Triton X-100, which dissolves the membranes without disrupting the v-ATPase–Ragulator–Rag interaction, completely suppressed the effect of amino acids or their esters to promote the binding of Raptor to RagB (Fig. 4B).

The PAT1 (SLC36A1) transporter is a proton-coupled amino acid transporter that localizes specifically to lysosomes (fig. S7A) and exports amino acids from HEK-293T cells expressing LAMP1-mRFP-FLAGX2. Lysosomes were either left intact or permeabilized with Triton X-100 or streptolysin O before measurement. Overexpression of PAT1 largely abolished amino acid accumulation inside lysosomes. Each value represents the mean ± SD of three independent samples. FLAG-RagB lysosomes were treated with DMSO or 2 μM SaA, activated with amino acid esters, and then incubated with Myc-Raptor. An organelar fraction from FLAG-metap2–expressing cells served as a negative control. (F) FLAG-RagB lysosomes were stimulated with amino acid esters in the presence of the proton ionophore FCCP or the nonhydrolyzable ATP analog AMP-PNP at 1 mM or 10 mM. Organelle samples were then incubated with Myc-Raptor cytosol, followed by FLAG immunoprecipitation and immunoblotting for Myc-Raptor and endogenous mTOR. (G) Model for inside-out activation of mTORC1 by lysosomal amino acids. The accumulation of amino acids inside the lysosomal lumen generates a signaling signal that is transmitted to the Rag GTPases via the v-ATPase–Ragulator interaction. In turn, the Rags physically recruit mTORC1 to the lysosomal surface.
out of the lysosomal lumen (23). In intact cells, overexpression of PAT1 completely suppressed mTORC1 activation by amino acids, and this effect was fully rescued by coexpression of constitutively active RagBGTP (Fig. 4C). In contrast, overexpression of PAT4 (SLC36A4), an amino acid transporter that does not localize to the lysosome, had no effect on mTORC1 activation by amino acids (fig. 5B, A and C).

These results strongly suggest that amino acid signaling begins inside the lysosome. Consistent with this possibility, stimulation of amino acid–starved 293T cells with 14C-amino acids led to the rapid appearance of labeled amino acids within lysosomes immunosolubilized through a FLAG-tagged lysosomal protein (Fig. 4D and fig. S6, A and B). Amino acid accumulation was reverted by lysosome permeabilization and largely prevented by PAT1 overexpression (Fig. 4D).

In the in vitro system, disruption of the v-ATPase by SalA or by shRNA against V0c blocked the amino acid–induced interaction of Raptor with RagB (Fig. 4E and fig. S5E). SalA inhibits the ATPase activity of V1 and the consequent rotation of the stalk and the V0 proteolipid subunits, blocked the amino acid–induced interaction between Raptor and RagB in a concentration–dependent manner (Fig. 4F and fig. S8D). Thus, ATP hydrolysis and the associated rotation of the v-ATPase appear to be essential to relay an amino acid signal from the lysosomal lumen to the Rag GTPases, whereas the capacity of the v-ATPase to set up the lysosomal proton gradient is dispensable in the in vitro system.

We propose a lysosome-centric inside-out model of amino acid sensing by mTORC1 in which amino acids must accumulate in the lysosomal lumen to initiate signaling (Fig. 4G). The v-ATPase is required for amino acid signaling to mTORC1 and functions between amino acids and the nucleotide loading of the Rag GTPases. Its placement in the pathway and its amino acid–sensitive interactions with the Rag-Ragulator complex implicate it as an essential component of the amino acid sensing mechanism.

References and Notes

Acknowledgments: We thank members of the Sabatini Lab as well as R. Perera for helpful suggestions, E. Spooner for the mass spectrometric analysis, and J. De Brabander (University of Texas Southwestern) for salicylihalamide A. Supported by grants from the National Institutes of Health (CA103866 and AI47389) and Department of Defense (W81XWH-07-0448) to D.M.S., awards from the W.M. Keck Foundation and LAM Foundation to D.M.S., and fellowship support from the Jane Coffin Childs Memorial Fund for Medical Research and the LAM Foundation to R.Z., from the Human Frontier Science Program to A.E., and from the Medical Scientist Training Program to S.W. D.M.S. is an investigator of the Howard Hughes Medical Institute.

Supporting Online Material
www.sciencemag.org/cgi/content/full/334/6056/678/DC1
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15 April 2011; accepted 13 September 2011
10.1126/science.1207056

RNAP II CTD Phosphorylated on Threonine-4 Is Required for Histone mRNA 3′ End Processing

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The RNA polymerase II (RNAPII) largest subunit contains a C-terminal domain (CTD) with up to 52 Tyr/Ser-Pro-Thr/Ser-Pro-Ser (24). Serine 2, 5, and 7 are known to be phosphorylated, and these modifications help to orchestrate the interplay between transcription and processing of messenger RNA (mRNA) precursors. Here, we provide evidence that phosphorylation of CTD Thr residues is required specifically for histone mRNA 3′ end processing, functioning to facilitate recruitment of 3′ processing factors to histone genes. Like Ser, Thr phosphorylation requires the CTD kinase CDK9 and is evolutionarily conserved from yeast to human. Our data thus illustrate how a CTD modification can play a highly specific role in facilitating efficient gene expression.

The carboxy-terminal domain (CTD) of the RNA polymerase II (RNAPII) largest subunit (Rpb1) consists of Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPSPS) consensus tandem repeats, which are conserved from yeast to human. The CTD, through phosphorylation on serine residues, links transcription to mRNA processing events (1–3). Ser phosphorylation by cyclin-dependent kinase 7, CDK7, a subunit of the general transcription factor TFIIH, and this modification functions to facilitate capping (4, 5). During transcriptional elongation, is phosphorylated by CDK9-P-TEFβ, which helps to coordinate RNA 3′ end processing and transcription termination (6, 7). Ser phosphorylation has been implicated in transcription and 3′ end processing of genes encoding certain small noncoding RNAs (8, 9). Tyr can also be phosphorylated by the c-Abl kinase (10). Although Thr has been reported to be phosphorylated in fission yeast (11), there is no evidence that this residue is modified in other species or what might the function of Thr be.

To investigate CTD function in a genetically tractable vertebrate cell system, we created an Rpb1 conditional knockout chicken cell line, DT40-Rpb1, by using methods developed previously to study other conserved proteins (12, 13) (fig. S1). These cells express, as the only source of Rpb1, a tetracycline (tet)-repressible cDNA encoding hemagglutinin (HA)–tagged human Rpb1 (human and chicken Rpb1 are 97% identical, and the CTD is very highly conserved among vertebrates; fig. S2). After addition of tet, Rpb1 became undetectable between 12 and 18 hours (Fig. 1A and fig. S3), and DT40-Rpb1 cells stopped growing.

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