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Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 25, Issue of June 24, pp. 23433–23436, 2005

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Received for publication, April 18, 2005,
and in revised form, May 3, 2005
Published, JBC Papers in Press, May 5, 2005,
DOI 10.1074/jbc.C500169200

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The removal of extracellular amino acids or leucine alone inhibits the ability of the mammalian target of rapamycin (mTOR) to signal to the raptor-dependent substrates, p70 S6 kinase and 4E-BP. This inhibition can be overcome by overexpression of the Rheb GTPase. Rheb binds directly to the amino-terminal lobe of the mTOR catalytic domain, and activates mTOR kinase in a GTP-dependent manner. Herein we show that the binding of Rheb to endogenous and recombinant mTOR is reversibly inhibited by withdrawal of all extracellular amino acids or just leucine. The effect of amino acid withdrawal is not attributable to changes in Rheb-GTP charging; amino acid withdrawal does not alter the GTP charging of recombinant Rheb. Moreover, the binding of mTOR to Rheb mutants that are unable to bind guanyl nucleotide *in vivo* is also inhibited by amino withdrawal. The inhibitory effect of amino acid withdrawal is exerted through an action on mTOR, at a site largely distinct from that responsible for the binding of Rheb; deletion of the larger, carboxyl-terminal lobe of the mTOR catalytic domain eliminates the inhibitory effect of amino acid withdrawal on Rheb binding, without altering Rheb binding *per se*. The lesser ability of the mTOR catalytic domain to bind Rheb after amino acid withdrawal does not persist after extraction and purification of the mTOR polypeptide. Amino acid withdrawl may generate an inhibitor of the Rheb-mTOR interaction that interferes with the signaling function of TOR complex 1.

The target of rapamycin (TOR)[1] is the founding member of the phosphatidylinositol 3′-OH kinase-related protein (Ser/Thr) kinase (PIKK) family (1). The TOR polypeptides in *Drosophila* and mammalian cells are now known to be major regulators of cell growth in part through their ability to phosphorylate and control the activity of the translational regulators, the p70 S6 kinases (S6Ks), and the 4E-BPs. TOR signaling is effected by two TOR-containing complexes; TOR phosphorylation of these translational regulators is mediated by a rapamycin-sensitive complex of TOR with the polypeptides LST8 and raptor, known as TOR complex 1 (2). LST8 binds to and stimulates the kinase activity of the TOR catalytic domain (3), whereas raptor binds the TOR substrates 4E-BP and p70 S6K and is critical for their effective phosphorylation by the TOR catalytic domain *in vivo* (4). In turn, signaling by TOR is controlled by multiple upstream inputs provided by receptor tyrosine kinases, through their control of PtdIns 3′OH kinase and the protein kinase B (PKB), by energy sufficiency through regulation of the AMP-activated protein kinase, and by amino acid sufficiency, whose effectors in this pathway are as yet unknown (5–7). Genetic evidence from *Drosophila*, fortified by genetic and biochemical data in mammalian systems, identified the critical regulators situated between PKB and TOR as the tuberous sclerosis complex (TSC), an obligatory heterodimer of the polypeptides Hamartin (TSC1) and Tuberin (TSC2), and Rheb, a Ras-like small GTPase. Rheb is a positive regulator of TOR signaling *in vivo*; the action of Rheb is opposed by the TSC complex, by virtue of the ability of the TSC complex to act as a Rheb GTPase activator, directly promoting the conversion of Rheb-GTP to Rheb-GDP. The inhibitory action of the TSC complex on Rheb is attenuated by PKB-catalyzed TSC2 phosphorylation, whereas the TSC-Rheb-GTPase activator activity is enhanced by AMP-activated protein kinase-catalyzed TSC2 phosphorylation (5–7). Thus the TSC complex is a major site at which RTKs and energy sufficiency control TOR signaling.

As regards the mechanisms by which Rheb acts as a positive regulator of TOR signaling, we recently demonstrated (8) that Rheb binds directly to the smaller, amino-terminal lobe of the mTOR catalytic domain, and the kinase activity of the TOR polypeptides bound to Rheb is determined by the state of Rheb nucleotide charging. TOR polypeptides bound to mutant Rhebs that are unable to bind any guanyl nucleotide are essentially devoid of protein kinase activity; conversely, TOR polypeptides bound to RhebQ64L, a mutant that is 90% GTP-bound *in vivo* (9), exhibit greater kinase activity than TOR polypeptides bound to wild type Rheb. Thus the mTOR polypeptide itself is a direct target of the Rheb GTPase. These findings do not preclude the operation of other Rheb effectors that may promote mTOR signaling indirectly, *e.g.* as by increasing intracellular amino acid levels. Notably, we also observed Rheb to be capable of interacting with LST8 and with raptor, independent of its ability to bind to TOR; this finding raised the possibility that, in addition to its ability to promote TOR catalytic activity, Rheb may also play a role in configuring TOR complex 1.

* This work was supported in part by National Institutes of Health (NIH) Grants DK17776 and CA73818 (to J. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

‡ Supported in part by a postdoctoral research fellowship award from the Massachusetts General Hospital Fund for Medical Discovery.

§ Both authors were supported in part by NIH Training Grant DK007028.

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[1] The abbreviations used are: TOR, target of rapamycin; mTOR, mammalian TOR; PKB, protein kinase B; TSC, tuberous sclerosis complex; S6K, S6 kinase; GST, glutathione *S*-transferase; D-PBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; GMPPNP, guanosine 5′-(β,γ-imido)triphosphate.

The mechanisms and site of action of amino acids in the control of TOR signaling and its relation to the mechanisms of Rheb action are poorly understood. Withdrawal of extracellular amino acids inhibits TOR signaling *in vivo* (10), as reflected by the progressive dephosphorylation of specific sites on p70 S6K (especially Thr^{412}, a major site of direct phosphorylation by mTOR; Ref. 11) and 4E-BP, over a period of 1–2 h. Although this response suggests an inhibition of TOR kinase activity, mTOR immunoprecipitates from amino acid-deprived cells exhibit kinase activity *in vitro* indistinguishable from that of mTOR isolated from amino acid-replete cells. Moreover, mutations of p70 S6K that eliminate the binding of the S6K polypeptide to raptor (when combined with a deletion of the S6K psuedosubstrate/autoinhibitory domain) render it resistant to dephosphorylation after amino acid depletion (as well as to rapamycin) (10). Together, these results suggest that amino acid withdrawal may not alter TOR catalytic activity but rather may interfere with the ability of the TOR catalytic domain to phosphorylate raptor-bound substrates. Whatever the mechanism by which amino acid withdrawal inhibits TOR signaling, this inhibition can be overcome by excess active (*i.e.* GTP-charged) Rheb. Thus, overexpression of recombinant Rheb can restore the phosphorylation of p70 S6K and 4E-BP despite the lack of extracellular amino acids; this effect of Rheb is inhibited by rapamycin (12–14).

In view of the finding that Rheb can interact directly with the components of TOR complex 1, we examined whether the interaction of Rheb with any of these elements is affected by amino acid sufficiency. Herein we show that the binding of recombinant Rheb to the mTOR catalytic domain *in vivo* is strongly inhibited by withdrawal of extracellular amino acids.

EXPERIMENTAL PROCEDURES

Reagents, antibodies, and all DNA constructs except mTOR-(2148–2300) and -(2148–2430) were described previously (8, 10). The latter two mTOR constructs were created by introduction of stop codons after the residues indicated into the mTOR-(2148–2549) construct. The anti-S6K (phospho-Thr^{412}) antibodies were purchased from Cell Signaling Technology, anti-FLAG M2 antibody from Sigma, and anti-GST monoclonal antibody from Santa Cruz Biotechnology.

Cell culture, transfection, and the procedures for amino acid or leucine withdrawal and readdtion are also described by Hara *et al.* (10); D-PBS contains the following components: $CaCl_2$ (0.1 g/liter), KCl (0.2 g/liter), KH_2PO_4 (0.2 g/liter), $MgCl_2$ + 6H2O (0.1 g/liter), NaCl (8 g/liter), Na_2HPO_4 + 7H2O (2.16 g/liter). Estimation of protein-protein interaction during transient expression and the measurement of Rheb guanyl nucleotide charging were performed are described by Long *et al.* (8).

RESULTS AND DISCUSSION

Withdrawal of extracellular amino acids results in the dephosphorylation of recombinant p70 S6K α1 at threonine 412 (equivalent to Thr^{389} in p70 S6K α2). Overexpression of wild type Rheb with S6K restores the phosphorylation of S6K(Thr^{412}) in a dose-dependent fashion, despite the withdrawal of extracellular amino acids (Fig. 1*A*). The ability of Rheb to restore S6K(Thr^{412}) phosphorylation requires that Rheb have an intact switch 1 and switch 2 domain and be capable of GTP charging *in vivo* (8). Moreover the effect of Rheb is sensitive to inhibition by rapamycin (Fig. 1*B*).

We recently showed that recombinant Rheb can bind directly to mTOR and that the protein kinase activity of mTOR is positively regulated by Rheb-GTP (8). Although the specific mechanism by which Rheb-GTP activates the mTOR kinase is not known, we inquired whether amino acid sufficiency affects the interaction of Rheb with mTOR. Recombinant GST-Rheb binds the endogenous mTOR complex 1 (Ref. 8 and Fig. 2*A*); here we show that prior amino acid withdrawal substantially reduces the recovery of endogenous mTOR with GST-Rheb;

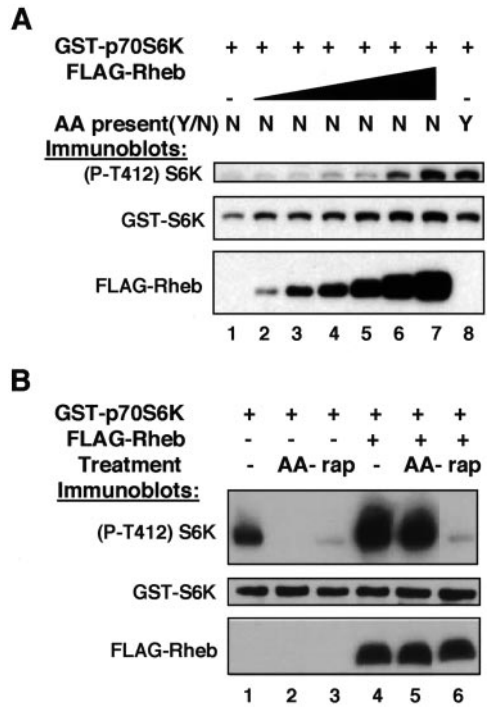

FIG. 1. **Rheb stimulates p70 S6K (Thr^{412}) phosphorylation and overcomes amino acid (*AA*) withdrawal-induced dephosphorylation.** *A*, HEK293T cells were transfected with pEBG-p70S6K (573 ng) and various amounts of pCMV5-FLAG-Rheb (31, 62.5, 125, 250, 500 ng, and 1 μg; *lanes 2–7*, respectively) or pCMV5-FLAG vector (1 μg, *lanes 1* and *8*). At 40 h post-transfection, the medium was changed to D-PBS for 2 h (*lanes 1–7*) or the cells were left untreated (*lane 8*). Cell lysates were analyzed by immunoblot. *B*, HEK293T cells were transfected with pEBG-p70S6K (*lanes 1–6*) with pCMV5-FLAG vector (*lanes 1–3*) or pCMV5-FLAG-Rheb (500 ng, *lanes 4–6*). At 40 h post-transfection, the cells were left untreated (*lanes 1*, *3*, *4*, and *6*), or the medium was changed to D-PBS (*lanes 2* and *5*). The cells were harvested 2 h later. Thirty minutes prior to harvest, rapamycin (*rap*, 50 nM, *lanes 3* and *6*) was added. Cell lysates were analyzed by immunoblot. *Y*, yes; *N*, no.

replacement of the usual medium, DMEM plus 10% serum, with D-PBS for 1.5 to 2 h greatly reduces the recovery of endogenous (Fig. 2*A*) or recombinant (Fig. 2*B*) mTOR with Rheb; removal of serum alone has no effect on the recovery of HA-mTOR with GST-Rheb (Fig. 2*B*, compare *lane 1* with *lane 3*), whereas the addition of an amino acid mixture alone to D-PBS is sufficient to fully restore the Rheb-bound mTOR to the levels recovered from cells in DMEM plus 10% serum (Fig. 2*B*, compare *lane 1* with *lane 4*). Recombinant Rheb binds to the carboxyl-terminal region of mTOR, specifically to the mTOR catalytic domain; moreover, independently of its ability to bind to mTOR, Rheb also binds directly to LST8 and to the carboxyl-terminal WD domains of raptor (8). We therefore compared the effect of amino acid withdrawal on the binding of GST-Rheb to these three polypeptides (Fig. 2*C*). Notably, whereas the binding of GST-Rheb to the mTOR carboxyl-terminal segment 2148–2549 is strongly inhibited by prior amino acid withdrawal, the binding of Rheb to LST8 and the raptor carboxyl terminus is unaffected. We showed previously that although removal of each of the nineteen amino acids gives some inhibition of mTOR signaling to p70 S6K, the most substantial inhibition seen upon withdrawal of a single amino acid occurs with removal of leucine, and removal of arginine is only slightly less inhibitory (10). Similarly we observe that the inhibitory effects on the Rheb-mTOR interaction caused by removal of all amino acids is largely reproduced by removal of leucine alone (Fig. 2*D*); a similar response is observed with arginine withdrawal (data not shown). As with its effect on mTOR signaling, the inhibitory effect of amino acid withdrawal

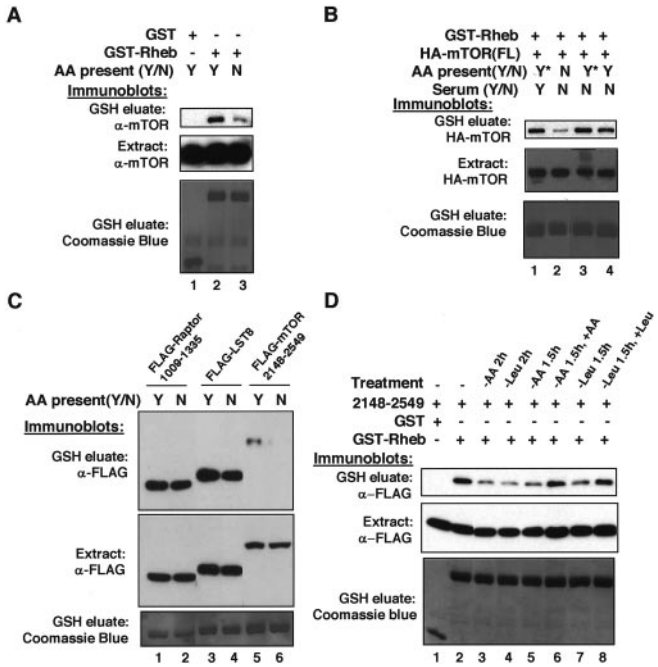

FIG. 2. **Binding of Rheb to mTOR is reversibly inhibited by withdrawal of extracellular amino acids (*AA*) or leucine.** *A*, HEK293T cells were transfected with pEBG (*lane 1*) or pEBG-Rheb wild type (*lanes 2* and *3*). Forty hours later, some cells (*lane 3*) were transferred to D-PBS, and all cells were harvested 2 h thereafter. Cells were extracted, and the GST fusion proteins were purified on GSH-Sepharose. The GSH eluates and aliquots of the extract were analyzed by immunoblot for mTOR (*top two panels*). A Coomassie Blue stain of the GSH eluate is in the *bottom panel*. *B*, pEBG-Rheb was cotransfected with pcDNA1-HA-mTOR in HEK293 cells. Forty hours later, some of the cells were transferred to D-PBS (*lane 2*) or DMEM without serum (*lane 3*) or to D-PBS containing 2× amino acid mixture (Invitrogen) (*lane 4*); the *asterisk* identifies cells incubated in DMEM. Cells were harvested 2 h later; GST-Rheb was isolated on GSH-Sepharose. After washing, the bound polypeptides (*top* and *bottom panels*), and aliquots of the cell extracts (*middle panel*) were subjected to SDS-PAGE and anti-HA immunoblot (*top* and *middle panels*) and Coomassie Blue stain (*bottom panel*). *C*, the effect of amino acid withdrawal on Rheb binding to raptor carboxyl-terminal segment 1009–1335, to LST8, and to the mTOR carboxyl-terminal segment 2148–2549. Transfection of pEBG-Rheb and pCMV5-FLAG constructs, extraction, GSH-Sepharose purification, and analysis were performed as described for *B*; some of the HEK293T cells were transferred from DMEM plus 10% fetal calf serum to D-PBS 2 h prior to harvest (*lanes 2*, *4*, and *6*). *D*, a comparison of the effect of withdrawal of leucine or all amino acids on Rheb binding to mTOR-(2148–2549). HEK293T cells were transfected with pCMV5-FLAG-mTOR-(2148–2549) and pEBG (*lane 1*) or pEBG-Rheb (*lanes 2–8*). After 40 h, some plates were transferred to D-PBS (*lanes 3*, *5*, and *6*) or to D-PBS containing a mixture of amino acids at a concentration equivalent to those present in DMEM, minus leucine (1× amino acids − leucine) (*lanes 4*, *7*, and *8*). Cells were harvested 1.5 h (*lanes 5* and *7*) or 2 h thereafter (*lanes 3*, *4*, *6*, and *8*). At 1.5 h after medium change, some cells incubated in D-PBS were refed with all amino acids (*lane 6*), whereas some of those incubated in "1× amino acids − leucine" were refed with leucine (*lane 8*) for another 30 min before harvest. Extraction, GSH-Sepharose purification, and analysis were performed as described for *B*. *Y*, yes; *N*, no.

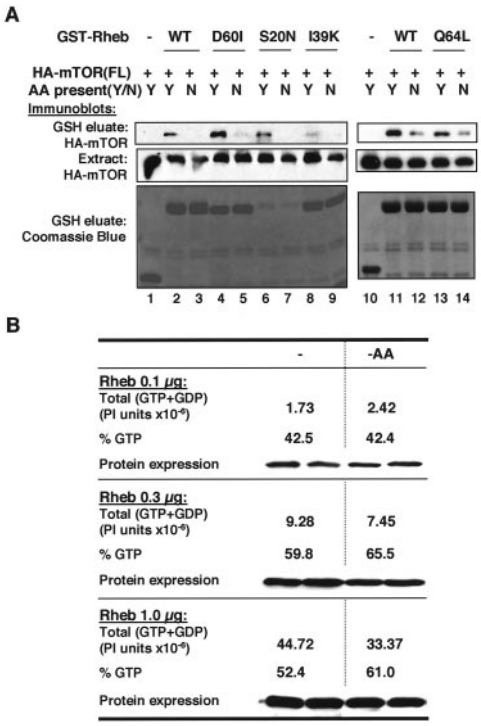

FIG. 3. **The inhibitory effect of amino acid (*AA*) withdrawal on the binding of HA-mTOR to Rheb is not due to a change in Rheb GTP charging.** *A*, pcDNA1 encoding HA-mTOR was coexpressed with wild type or mutant Rhebs, each expressed as a GST fusion protein. After 40 h, some of the cells were transferred to D-PBS (*lanes 3*, *5*, *7*, *9*, *12*, and *14*); all plates were extracted 2 h later. The GST-Rheb polypeptides were purified and washed on GSH-Sepharose and the eluates (*top* and *bottom panels*), and aliquots of the extracts (*middle panels*) were subjected to SDS-PAGE and anti-HA immunoblot (*top* and *middle panels*) or Coomassie Blue stain (*bottom panels*). *B*, Rheb GTP charging during transient expression. Amino acid withdrawal was accomplished by transfer to D-PBS with $^{32}P_i$ 2 h prior to harvest. The *insets* show images obtained from a single immunoblot of the GST-Rheb polypeptides analyzed for each of the treatments indicated. *Y*, yes; *N*, no.

on the Rheb-mTOR interaction is reversible; readdition of amino acids 30 min prior to harvest largely restores mTOR binding to Rheb (Fig. 2*D*). A similar reversibility is observed with regard to leucine regulation of Rheb-mTOR binding (Fig. 2*D*). Thus the amino acid regulation of the Rheb-TOR association displays a specificity similar to the amino acid regulation of mTOR signaling.

A novel feature of the binding of Rheb to its effector mTOR, and one that contrasts with the interaction of other Ras-like GTPases with their known effectors, is that the Rheb-mTOR interaction does not require Rheb GTP charging (8). Moreover nucleotide-deficient Rheb polypeptides bind more tightly to mTOR than does nucleotide-replete Rheb, and Rheb-GTP charging, although it promotes mTOR kinase activity *in vivo*, actually diminishes the strength of the Rheb-mTOR interaction *in vivo* and *in vitro* (8). Notably, amino acid withdrawal interferes with the ability of mTOR to bind to wild type Rheb and to the nucleotide-deficient, switch 1 and switch 2 mutants of Rheb to a similar extent (Fig. 3*A*); in addition, the binding of these nucleotide-deficient Rheb mutants to the mTOR fragments (2148–2549) is also potently inhibited by amino acid withdrawal (supplemental Fig. 1). Reciprocally, amino acid withdrawal also diminishes the binding of mTOR to Rheb(Q64L), a mutant that exhibits nearly 90% GTP charging *in vivo* (Fig. 3*A*). This indicates that the effect of amino acid withdrawal is not mediated by changes in Rheb guanyl nucleotide charging. In fact, amino acid withdrawal does not appreciably alter the fractional guanyl nucleotide charging of wild type recombinant Rheb (Fig. 3*B*) as observed by Zhang *et al.* (15), but in contrast to the findings of Smith *et al.* (16).

We next examined the effect of further deletion of the mTOR carboxyterminal fragment on the ability of amino acids to regulate Rheb binding; the mTOR fragments 2148–2430 and 2148–2300 each bind wild type Rheb comparably with to mTOR-(2148–2549); however, the binding of Rheb to the 2148–2300 fragment is not inhibited by prior amino acid withdrawl, whereas the binding of Rheb to the other two mTOR fragments shows similar inhibition by amino acid withdrawal (Fig. 4*A*). This result establishes that the regulation of the mTOR-Rheb

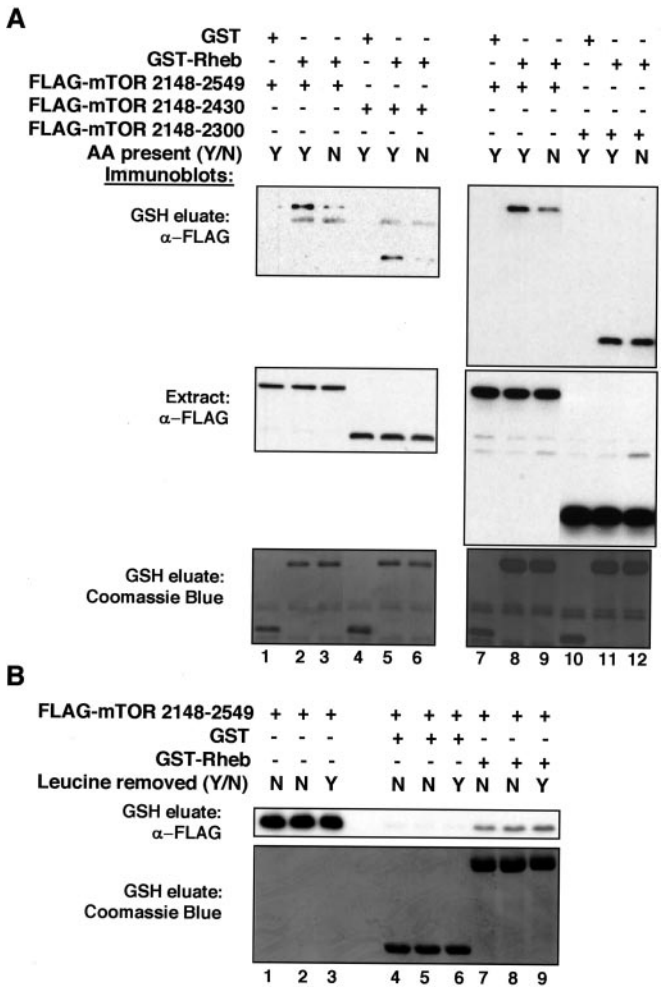

FIG. 4. **The inhibitory effect of amino acid (*AA*) withdrawal on Rheb-TOR interaction is exerted on TOR through a site that is distinct from the amino-terminal lobe of TOR catalytic domain.** *A*, HEK293T cells were cotransfected with pCMV5-FLAG-mTOR-(2148–2549) (*lanes 1–3* and *7–9*) or -(2148–2430) (*lanes 4–6*) or -(2148–2300) (*lanes 10–12*) and pEBG (*lanes 1*, *4*, *7*, and *10*) or pEBG-Rheb (*lanes 2*, *3*, *5*, *6*, *8*, *9*, *11*, and *12*). After 40 h, some plates were transferred to D-PBS (*lanes 3*, *6*, *9*, and *12*). Extraction, GSH-Sepharose purification, and analysis were preformed as described in the legend to Fig. *2B*. *B*, the FLAG-tagged mTOR fragment 2148–2549 was expressed in HEK293T cells; some cells were starved for leucine (*lanes 3*, *6*, and *9*) for 2 h before harvest. The mTOR fragment was extracted using 0.1 M NaF, immunopurified, and eluted from immobilized anti-FLAG monoclonal antibody. Aliquots of FLAG-mTOR-(2148–2549) were incubated *in vitro* with GSH-Sepharose-immobilized GST (*lanes 4–6*) or GST-Rheb (*lanes 7–9*) also purified after transient expression and charged *in vitro* with GMPPNP. After washing, the polypeptides retained on GSH-Sepharose were analyzed by anti-FLAG immunoblot (*upper panel*) and Coomassie Blue stain (*lower panel*). An aliquot representing 20% of the FLAG-polypeptide loaded with the GST proteins is shown in *lanes 1–3*. *Y*, yes; *N*, no.

interaction by amino acid sufficiency is exerted primarily and perhaps exclusively through an effect on mTOR, through the segment 2301–2430, which corresponds to the larger, carboxyl-terminal lobe of the mTOR catalytic domain. We next inquired as to whether the ability of amino acid sufficiency to regulate the ability of the mTOR-(2148–2549) fragment to bind Rheb is retained *in vitro*. FLAG-tagged mTOR-(2148–2549) was expressed in HEK293 cells, a portion of which were subjected to amino acid withdrawal prior to harvest. The cells were extracted in the presence of 0.1 M NaF; after anti-FLAG immunoaffinity purification, the extensively purified preparations of FLAG-mTOR-(2148–2549) were incubated with immobilized GST or GST-Rheb charged with GMPPNP. After brief washing, the retained FLAG-mTOR polypeptides were analyzed by FLAG immunoblot. As shown in Fig. 4*B*, GST-Rheb binds FLAG-mTOR-(2148–2549) specifically, however, in equal amounts whether the mTOR polypeptides are extracted from amino acid-replete or -deficient cells. Thus the ability of amino acid withdrawal to inhibit the ability of mTOR-(2148–2549) to bind to Rheb is not due to a stable modification of the mTOR polypeptide that survives cell extraction.

The molecular mechanism by which amino acid withdrawal acts upon the carboxyl-terminal lobe of the mTOR catalytic domain to interfere with Rheb binding to the adjacent amino-terminal lobe is not known. As with mTOR kinase activity (10), the failure of the inhibitory effect of amino acid withdrawal to survive cell disruption argues against a mechanism that involves stable modifications of the mTOR polypeptide. Rather, this behavior suggests that the *in vivo* inhibition of mTOR signaling and the Rheb/mTOR interaction caused by amino acid withdrawal is due to the generation of an inhibitor that binds to the mTOR catalytic domain and interferes by a non-covalent mechanism with the mTOR-catalyzed S6K phosphorylation within the TOR complex 1, as well as with the ability of mTOR to bind Rheb. The putative inhibitor is lost on cell disruption, thereby restoring the mTOR kinase activity assayed *in vitro*, as well as the ability of the mTOR catalytic domain to bind added Rheb *in vitro*. The inhibitory effect of amino acid withdrawal on mTOR signaling may be due directly to the inhibition of Rheb binding to mTOR; if so the ability of overexpressed Rheb to overcome the inhibitory effect of amino acid withdrawal may be due simply to flooding the cell with an excess of Rheb-GTP that is sufficient to overcome the effect of the inhibitor. The ability of Rheb to bind to raptor and LST8, the other components of the TOR complex 1, may also be relevant to the mechanism of Rheb action within the TORC1. Amino acid withdrawal has been reported previously to increase the association of raptor with mTOR but only in complexes that contain LST8 (3, 17); whether that phenomenon is mechanistically related to the ability of amino acid withdrawal to inhibit Rheb binding to mTOR is unknown.

Acknowledgment—We thank K. Yonezawa for useful discussions.

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J. Biol. Chem. 2005, 280:23433-23436.
doi: 10.1074/jbc.C500169200 originally published online May 5, 2005