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Rheb Binding to Mammalian Target of Rapamycin (mTOR) Is Regulated by Amino Acid Sufficiency*

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The target of rapamycin (TOR) 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 Raptor GTPase activator, directly promoting the conversion of Raptor-GTP to Raptor-GDP. The inhibitory action of the TSC complex on Rheb is attenuated by PKB-catalyzed TSC2 phosphorylation, whereas the TSC-Tuberin-Rheb complex 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 TOR 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 Rheb64L, a mutant that is 90% GTP-bound in vivo (9), exhibit greater kinase activity than TOR polypeptides bound to wild type Rheb. Thus the TOR polypeptide itself is a direct target of the Rheb GTPase. These findings do not preclude the operation of other Rheb effectors that may promote TOR 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.
Amino Acid Regulation of Rheb-mTOR Binding in Vivo

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 Thr412, 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 pseudosubstrate/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-Thr412) 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 readdition are also described by Hara et al. (10). D-PBS contains the following components: CaCl2 (0.1 g/liter), KCl (0.2 g/liter), KH2PO4 (0.2 g/liter), MgCl2 2H2O (0.2 g/liter), NaH2PO4 (0.1 g/liter), NaCl (8 g/liter), Na2HPO4, 7H2O (2.16 g/liter). Estimation of protein-protein interaction during transient expression and the measurement of Rheb guanyl nucleotide charging were performed as 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 Thr389 in p70 S6K α2). Overexpression of wild type Rheb with S6K restores the phosphorylation of S6K(Thr412) in a dose-dependent fashion, despite the withdrawal of extracellular amino acids (Fig. 1A). The ability of Rheb to restore S6K(Thr412) 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. 1B).

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 inquire whether amino acid sufficiency affects the interaction of Rheb with mTOR. Recombinant GST-Rheb binds the endogenous mTOR complex 1 (Ref. 8 and Fig. 2A); here we show that prior amino acid withdrawal substantially reduces the recovery of endogenous mTOR with GST-Rheb.
whereas some of those incubated in D-PBS were refed with all amino acids (lane 6). 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) were subjected to SDS-PAGE and anti-HA immunoblot (top and middle panels) or Coomassie Blue stain (bottom panel). B, the effect of amino acid withdrawal on Rheb binding to raptor carboxyl-terminal segment 2148–2549. HEK293T cells were transfected with pEBG (lane 5) or pEBG-Rheb (lane 8). At 1.5 h after medium change, some of the cells were transferred to D-PBS (lanes 3, 5, and 6) or to D-PBS containing a mixture of amino acids at a concentration 2×, amino acid mixture (Invitrogen); some of those incubated in D-PBS were refed with all amino acids (lane 6). After 40 h, some of the cells were transferred to D-PBS (lanes 2) or DMEM without serum (lanes 7–9). 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 mTOR carboxyl-terminal segment 1009–1335, to LST8, and to the mTOR-(2148–2549). HEK293T cells were transfected with 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 DMEM containing 2× amino acid mixture (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.

on the Rheb-mTOR interaction is reversible; readdition of amino acids 30 min prior to harvest largely restores mTOR binding to Rheb (Fig. 2D). A similar reversibility is observed with regard to leucine regulation of Rheb-mTOR binding (Fig. 2D). 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. 3A); in addition, the binding of these nucleotide-deficient Rheb mutants to the mTOR fragments (2148–2549) is also potentely 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. 3A). 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. 3B) 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 withdrawal, whereas the binding of Rheb to the other two mTOR fragments shows similar inhibition by amino acid withdrawal (Fig. 4A). This result establishes that the regulation of the mTOR-Rheb binding displays a specificity similar to the amino acid regulation of the mTOR-Rheb interaction.
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) and pEG-B-Rheb. After 40 h, some plates were transferred to D-PBS (lanes 3, 6, and 9) and Coomassie Blue stain (lower panel) and Coomassie Blue stain (upper panel) were analyzed by anti-FLAG immunoblot. As shown in lanes 4–6, GST-Rheb binds 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 6K 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.

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REFERENCES

1. Jacinto, E., and Hall, M. N. (2003) Nat. Rev. Mol. Cell Biol. 4, 117–128
2. Martin, D. E., and Hall, M. N. (2005) Curr. Opin. Cell Biol. 17, 158–166
3. Kim, Do-Hung, Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V. P., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2000) Mol. Cell 11, 895–904
4. Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, H., Hidayat, S., Yoshino, K., Harai, K., Avruch, J., and Yonezawa, K. (2003) J. Biol. Chem. 278, 15461–15464
5. Hay, N., and Sonenberg, N. (2004) Genes Dev. 18, 1926–1945
6. Li, Y., Corradetti, M. M., Inoki, K., and Guan, K.-L. (2003) Trends Biochem. Sci. 28, 32–38
7. Findlay, G. M., Harrington, L. S., and Lamb, R. F. (2005) Curr. Opin. Genet. Dev. 15, 69–76
8. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005) Curr. Biol. 15, 702–713
9. Li, Y., Inoki, K., and Guan, K. L. (2004) Mol. Cell 15, 797–805
10. Harai, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C., and Avruch, J. (1999) J. Biol. Chem. 272, 14484–14494
11. Iestani, S., Harai, K., Tokunaga, C., Inoue, H., Avruch, J., and Yonezawa, K. (1999) J. Biol. Chem. 274, 34493–34498
12. Sauedco, L. J., Gao, X., Xiang, L. G., Li, H., Pan, D., and Edgar, B. A. (2003) Nat. Cell Biol. 5, 586–591
13. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, F., Daram, P., Breuer, S. Thomas, G., and Hafen, E. (2003) Nat. Cell. Biol. 5, 559–565
14. Aspurgia, P. J., and Tamanoi, F. (2004) Cell. Signal. 16, 1105–1112
15. Zhang, Y., Gao, X., Sauedco, L. J., Xu, B., Edgar, B. A., and Pan, D. (2003) Nat. Cell Biol. 5, 578–581
16. Smith, E. M., Finn, S. T., Tee, A. R., Browne, G. J., and Proud, C. G. (2004) J. Biol. Chem. 280, 18717–18727
17. Kim, Do-Hung, Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002) Cell 110, 163–175
