Activation of mTORC1 in two steps: Rheb-GTP activation of catalytic function and increased binding of substrates to raptor

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Abstract

The signalling function of mTOR complex 1 is activated by Rheb-GTP, which controls the catalytic competence of the mTOR (mammalian target of rapamycin) kinase domain by an incompletely understood mechanism. Rheb can bind directly to the mTOR kinase domain, and association with inactive nucleotide-deficient Rheb mutants traps mTOR in a catalytically inactive state. Nevertheless, Rheb-GTP targets other than mTOR, such as FKBP38 (FK506-binding protein 38) and/or PLD1 (phospholipase D1), may also contribute to mTOR activation. Once activated, the mTOR catalytic domain phosphorylates substrates only when they are bound to raptor (regulatory associated protein of mTOR), a separate polypeptide within the complex. The mechanism of insulin/nutrient stimulation of mTOR complex 1 signalling, in addition to Rheb-GTP activation of the mTOR catalytic function, also involves a stable modification of the configuration of mTORC1 (mTOR complex 1) that increases access of substrates to their binding site on the raptor polypeptide. The mechanism underlying this second step in the activation of mTORC1 is unknown.

Introduction

The protein kinase TOR (target of rapamycin), is a fundamental regulator of cellular metabolism, growth and proliferation in all eukaryotes [1], functioning through two largely independent multiprotein complexes. TORC (TOR complex) 1 contains the polypeptides raptor (regulatory associated protein of TOR) and Lst8, and controls cell growth through substrates that regulate transcriptional, translational and post-translational processes. TORC2 contains the polypeptides Lst8, rictor (rapamycin-insensitive companion of TOR) and sin1, and, in some organisms, other less well conserved polypeptides; TORC2 controls some aspects of cell proliferation and metabolism and is especially concerned with the co-ordination of the cytoskeleton with cell enlargement and cell division. We have been particularly interested in the mechanisms that underlie the activation of mTORC1 (mammalian TORC1) [2], and, in the present paper, we summarize the status of information on this topic as well as ongoing work in this laboratory.

Key words: insulin, leucine, mammalian target of rapamycin (mTOR), regulatory associated protein of mTOR (raptor), Rheb, tuberous sclerosis complex (TSC).

Abbreviations used: 4E-BP, eukaryotic initiation factor 4E-binding protein; FKBP, FK506-binding protein; GAP, GTPase-activating protein; IGF-I, insulin-like growth factor 1; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PRAS40, proline-rich Akt substrate of 40 kDa; raptor, regulatory associated protein of target of rapamycin; Rheb, Schizosaccharomyces pombe Rheb; Sin1, Schizosaccharomyces pombe target of rapamycin; S6K1, S6 kinase 1; TSC, target of rapamycin; TSC, TOR complex; TSC, tuberous sclerosis complex.

1Dedicated to our deceased colleagues K. Yonezawa and J.C. Lawrence, Jr.

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Insulin and growth factors stimulate signalling by mTORC1, whereas various forms of stress, especially hypoxia and energy depletion, are inhibitory. Genetic evidence from Drosophila established unequivocally that the Ras family GTPase Rheb is an indispensable activator of TORC1 in the insulin/IGF-I (insulin-like growth factor 1) receptor pathway. Moreover, Rheb acts epistatically to the inhibitory action of the TSC (tuberous sclerosis complex) 1–TSC2 heterodimer on TORC1 signalling, a relationship explained by the finding that TSC is an activator of the Rheb GTPase activity, i.e. a Rheb–GAP (GTPase-activating protein) [3]. Studies in mammalian cells rapidly demonstrated the conservation of these relationships, and identified the TSC as a major site of regulation of TORC1 activity [4]. Insulin/IGF-I receptor/PI3K (phosphoinositide 3-kinase) activation of Akt inhibits TSC–GAP function, whereas hypoxia and energy depletion, through the transcriptional up-regulation of REDD (regulated in development and DNA damage responses) and activation of AMPK (AMP-activated protein kinase)/GSK3 (glycogen synthase kinase 3), disinhibit the TSC–GAP activity to lower Rheb-GTP. Cells lacking a functional TSC–Rheb–GAP exhibit constitutive activation of TORC1 signalling which is not increased further by insulin, or effectively inhibited by hypoxia or metabolic stress, but is inhibited by depletion of Rheb. TORC1 signalling is also regulated by amino acids; removal of extracellular amino acids, most ubiquitously and potently leucine, inactivates TORC1 signalling [5]. The site of action of leucine appears to be distal to the TSC; TORC1 signalling in TSC-deficient cells,
although highly resistant to inhibition by hypoxia/metabolic stress, is hardly diminished in its susceptibility to inhibition by leucine withdrawal. Unlike insulin or energy depletion, amino acid withdrawal does not significantly alter Rheb-GTP charging. Nevertheless, the inhibition of TORC1 signalling caused by leucine withdrawal can be completely reversed by overexpression of recombinant Rheb. The ability of excess Rheb-GTP to restore TORC1 activity in leucine-deprived cells suggests that leucine withdrawal somehow interferes with Rheb-GTP activation of TORC1 [2].

Rheb activation of TOR catalytic function

Seeking the mechanism of Rheb regulation of mTORC1, we observed that endogenous mTORC1 binds to transiently expressed Rheb, much better than to V12Ras or V12Rap1 expressed at comparable levels [6]. This raised the possibility that Rheb regulates mTORC1 through a direct interaction with the mTOR (mammalian TOR) polypeptide. Rheb binds specifically to the upper lobe of the mTOR catalytic domain in vivo and in vitro; however, in contrast with all known GTPase–effector interactions, the mTOR–Rheb interaction is not enhanced by Rheb-GTP charging. Moreover, a variety of inactive Rheb switch 1 and switch 2 [7] mutants are relatively unaltered in their ability to bind to mTOR and the inactive Rheb mutants, S20N and D60I, which are completely unable to bind guanyl nucleotides in vivo or in vitro, bind to mTOR much more tightly than does wild-type Rheb. Nevertheless, whereas the mTOR polypeptides that co-precipitate with wild-type Rheb exhibit clear-cut kinase activity towards 4E-BP (eukaryotic initiation factor 4E-binding protein) and an S6K1 (S6 kinase 1) fragment in vitro, similar amounts of mTOR polypeptide retrieved with S20N or D60I mutant Rheb are essentially devoid of kinase activity, assayed in vitro. A reciprocal situation is observed with the Rheb mutant Q64L, which achieves ∼90% GTP charging during transient expression, compared with ∼50% for wild-type Rheb. Although mTOR binds more weakly to N64L mutant Rheb than to wild-type Rheb during transient expression, the mTOR polypeptides retrieved with Q64L mutant Rheb exhibit approx. 2-fold higher kinase-specific activity. On the basis of these findings, we infer that a direct interaction of mTORC1 with Rheb-GTP is necessary for the acquisition of kinase activity. Rheb-GTP charging does not promote the Rheb–mTOR interaction, but GTP-charged Rheb is required for activation of mTOR catalytic activity. Although an association of endogenous Rheb with mTOR has not been observed in mammalian cells, the work of Tamanoi and colleagues in Schizosaccharomyces pombe is strongly supportive of the functional significance of a direct Rheb–TOR interaction. Urano et al. [8] identified a series of hyperactive mutants of SpRheb (S. pombe Rheb), and observed that, whereas wild-type SpRheb exhibited little association with endogenous SpTOR (S. pombe TOR), the hyperactive Rheb mutant K120R co-sedimented entirely with SpTOR on sucrose density gradient centrifugation.

At least two other mechanisms by which Rheb-GTP can activate mTORC1 have been proposed. Thus Bai et al. [9] have reported that FKBP (FK506-binding protein) 38 binds to the mTOR FKBP/rapamycin-binding domain, a segment of mTOR located just N-terminal to the catalytic domain, and inhibits mTOR kinase activity. Rheb binds specifically to FKBP38 in a GTP-dependent manner through its switch 1 segment [10] and displaces FKBP38 from the FKBP/rapamycin-binding domain, relieving the inhibition of the kinase domain. Sun et al. [11] report that Rheb-GTP binds and activates PLD1 (phospholipase D1) to generate phosphatic acid, a positive regulator of mTOR signalling [12] that also binds to the mTOR FKBP/rapamycin-binding domain. The contribution of these mechanisms to mTOR activation by Rheb-GTP relative to the direct interaction of Rheb-GTP with mTOR remains to be defined. Considerable additional work is needed to elucidate the mechanisms by which the Rheb–TOR interaction promotes TOR kinase activity; e.g. is a direct Rheb–mTOR association necessary just for the acquisition of mTOR kinase activity or for its maintenance as well? Are there other polypeptides necessary for Rheb binding to or regulation of mTOR? What is the structural basis for the activation of the mTOR catalytic domain by Rheb-GTP?

The insulin/IGF-I pathway controls Rheb-GTP charging through regulation of the GTPase-activating function of the TSC. In contrast, amino acids appear to control the efficacy of Rheb–GTP towards mTOR. Seeking the mechanism for this control, we observed that removal of extracellular amino acids or just leucine inhibits the association of mTOR with both wild-type and inactive nucleotide-deficient Rheb during transient expression [13]. This finding raised the possibility that the inhibitory effect of amino acid withdrawal on mTOR complex 1 signalling in vivo is attributable to a loss of the Rheb–mTOR interaction; moreover, it suggested that the ability of excess recombinant Rheb to overcome this inhibition is attributable simply to swamping the cell with Rheb-GTP and restoring, by mass action, the mTOR–Rheb–GTP interaction. As to the mechanism by which amino acid withdrawal interferes with the Rheb–mTOR interaction, it appears entirely attributable to an action on mTOR rather than Rheb, at a site on mTOR largely distinct from that which mediates the binding of Rheb. Thus deletion of the larger C-terminal lobe of the mTOR catalytic domain eliminates the inhibitory effect of amino acid withdrawal on Rheb binding to the upper lobe of the catalytic domain, without altering Rheb binding [13]. The lesser ability of the mTOR catalytic domain to bind Rheb in vivo after amino acid withdrawal does not persist after extraction and purification of the mTOR polypeptide, suggesting that it does not reflect some covalent modification of mTOR. The recent findings of Sancak et al. [14] that a Rag (Ras-related GTPase) heterodimer binds to the raptor polypeptide in an amino-acid-dependent manner, and thereby recruits mTORC1 to the endomembrane compartment that contains Rheb, provides a plausible molecular mechanism by which amino acids can regulate the Rheb–mTOR interaction.
Regulation of raptor substrate binding

TORC1 exhibits a feature that is uncommon, if not unique among protein kinases; the two major functions of the kinase, namely substrate binding and catalysis of phosphate transfer, are assigned to two separate polypeptides. The TOR protein is the catalytic effector, through the PI3K-related catalytic domain located in its C-terminal region, whereas protein substrate binding occurs on raptor. Recognition of this feature emerged from efforts to understand the differential sensitivity of the mTOR kinase activity, as measured in vitro, to extraction with various detergents. As first reported by Brunn et al. [15] extraction with the relatively weak detergent Tween 20 enables visualization of a robust kinase activity in vitro towards the in vivo substrate, 4E-BP; in contrast, comparable amounts of mTOR polypeptide retrieved by extraction in Triton X-100 or Nonidet P40 are virtually devoid of kinase activity in vitro towards the relevant sites on 4E-BP, although mTOR autophosphorylation activity is actually enhanced [16]. Hara et al. [17] used detergent-free methods to isolate mTOR with a fully functional kinase activity towards 4E-BP, and, in so doing, retrieved the heterotrimer now generally recognized as mTORC1 (contemporaneously with Kim et al. [18] and Loewith et al. [19]). Hara et al. [17] showed that the mTORC1 substrates S6K1 and 4E-BP bind directly to raptor and that the detergent CHAPS, in a dose-dependent manner, reduced the mTORC1 4E-BP kinase activity in parallel with removal of the raptor polypeptide from the mTOR–Lst8 heterodimer. The interaction of substrates with raptor was illuminated further by the finding that the TOS (TOR signalling) motif (Phe–Ac–φ–Ac–φ, where Ac is glutamic acid/aspartic acid and φ is leucine/isoleucine/methionine) on S6K1 and 4E-BP, which is necessary for their rapamycin-sensitive phosphorylation in vivo [20], is necessary for their direct binding to raptor in vitro [21].

The occurrence of a shared motif on the mTOR substrates S6K1 and 4E-BP {as well as on STAT3 {signal transducer and activator of transcription 3} [22]} suggests that these substrates bind to a common (or overlapping) site(s) on raptor. Early work demonstrated that overexpression of S6K1 inhibited the mTORC1-catalysed phosphorylation of 4E-BP [23] and, more recently, several groups reported that the mTORC1 substrates S6K1, 4E-BP and PRAS40 (proline-rich Akt substrate of 40 kDa) were each mutually competitive in their binding to raptor [24–27]. More importantly, RNAi (RNA interference)-induced depletion of endogenous PRAS40 in several cell types is observed to enhance insulin–or amino-acid-stimulated, mTORC1-catalysed phosphorylation of S6K1 and 4E-BP [27]. The finding that depletion of one mTORC1 substrate enhances the mTORC1-catalysed phosphorylation of others suggests that even during insulin/nutrient stimulation, the availability of substrate-binding sites on endogenous raptor is limiting for mTORC1-catalysed substrate phosphorylation. Wang et al. [28] provided the most direct evidence that raptor-mediated substrate binding is regulated, demonstrating that the activation of mTORC1 signalling by insulin treatment of 3T3-L1 adipocytes is accompanied by an increased ability of the extracted mTORC1 complex to bind to the 4E-BP polypeptide in vitro. We also find that inhibition of PI3K or amino acid withdrawal before cell extraction each results in an inhibition in the ability of the extracted mTORC1 to bind 4E-BP. However, this regulation of substrate binding to raptor is only evident using the intact mTORC1; if raptor is extracted with Triton X-100, so as to remove it from the mTOR–Lst8 heterodimer, its ability to bind substrates is enhanced, as compared with a similar amount of raptor embedded in the mTORC1, and is unaffected by the nature of cell treatment before extraction.

Thus activation of mTORC1 involves two distinguishable steps: first, a Rheb-GTP-induced activation of the mTOR catalytic function, and, secondly, a reconfiguration of the intact complex that enhances access to and/or affinity for substrates. Although the substrate-binding site is entirely on raptor, the regulation of substrate binding requires an intact mTOR–raptor complex. The persistence of the altered substrate binding after extraction of the complex from the cell is not due to an alteration in the content of co-purifying raptor-associated polypeptides, such as PRAS40, but may reflect some stable modification of mTOR or both. A number of raptor phosphorylation sites have been identified, and their relationship to altered substrate binding, as well as the requirement for an active mTOR kinase polypeptide, are currently under investigation.

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