Rheb and Rags come together at the lysosome to activate mTORC1

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Abstract

mTORC1 (mammalian target of rampamycin complex 1) is a highly conserved protein complex regulating cell growth and metabolism via its kinase mTOR (mammalian target of rapamycin). The activity of mTOR is under the control of various GTPases, of which Rheb and the Rags play a central role. The presence of amino acids is a strict requirement for mTORC1 activity. The heterodimeric Rag GTPases localize mTORC1 to lysosomes by their amino-acid-dependent interaction with the lysosomal Ragulator complex. Rheb is also thought to reside on lysosomes to activate mTORC1. Rheb is responsive to growth factors, but, in conjunction with PLD1 (phospholipase D1), is also an integral part of the machinery that stimulates mTORC1 in response to amino acids. In the present article, we provide a brief overview of novel mechanisms by which amino acids affect the function of Rags. On the basis of existing literature, we postulate that Rheb is activated at the Golgi from where it will travel to lysosomes. Maturation of endosomes into lysosomes may be required to assure a continuous supply of GTP-bound Rheb for mTORC1 activation, which may help to drive the maturation process.

Introduction

mTORC1 (mammalian target of rapamycin complex 1) is a major regulator of cell growth and metabolism, controlling diverse processes including protein synthesis, ribogenesis, mitochondrial capacity and autophagy [1]. In mammals, mTORC1 consists of the serine/threonine kinase mTOR (mammalian target of rapamycin), mLST8 (mammalian lethal with sec-13 protein 8), raptor (regulatory associated protein of mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa). Raptor functions both as a scaffolding protein by interacting with mTOR substrates and as a regulatory unit for mTOR. The role of mLST8 is less well understood. The crucial function of mTOR requires sophisticated mechanisms of regulation that will result in activity levels tailored to meet the metabolic requirements of a cell under various conditions. Activation of mTORC1 seen following stimulation of cells with growth factors and especially the insulin pathway has been well characterized. Active mTORC1 has multiple substrates that include p70 S6 kinase and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1), which stimulate protein translation via distinct mechanisms [2]. In contrast, various cellular stresses such as nutrient deprivation or hypoxia strongly decrease mTORC1 activity. In general, the signalling pathways that sense cellular stress are dominant over growth factor signalling, thus preventing cell growth under unfavourable conditions. A classical example is the observation by Hara et al. [3] that insulin-mediated activation of mTORC1 is prevented in cells depleted of amino acids. Remarkably, lack of amino acids does not interfere in the activation of upstream elements of the insulin pathway such as the insulin receptor itself or the serine/threonine kinase PKB (protein kinase B). In the presence of amino acids, PKB activates mTOR indirectly by inhibiting TSC (tuberous sclerosis complex) 1/2 [4,5]. This complex consists of TSC1 and TSC2, loss of which results in tuberous sclerosis, a disease characterized by growth of non-malignant tumours in multiple organs. TSC2 contains a GAP (GTPase-activating protein) domain and functions as a negative regulator of the Ras-like GTPase Rheb [6,7]. Like other Ras-like GTPases, Rheb is only active (i.e. functions as an activator of mTORC1) when bound to GTP. Cells lacking either TSC1 or TSC2 have a high level of GTP-bound Rheb, which results in growth-factor-independent mTORC1 activity. However, when these cells are amino-acid-depleted, mTORC1 activity is abolished and this is not accompanied by a decrease in GTP-bound Rheb [8,9]. This indicates that, next to Rheb, mTORC1 requires a second input for its activity that signals amino acid sufficiency.

Key words: amino acid, lysosome, mammalian target of rapamycin complex 1 (mTORC1), Rag, Rheb.

Abbreviations used: 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GEF, guanine-nucleotide-exchange factor; LRS, leucyl-tRNA synthetase; MAPK, mitogen-activated protein kinase; mLST8, mammalian lethal with sec-13 protein 8; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; PA, phosphatidic acid; PAT1, proton-coupled amino acid transporter 1; PKB, protein kinase B; PLD1, phospholipase D1; PRAS40, proline-rich Akt substrate of 40 kDa; PX, Phox homology; raptor, regulatory associated protein of mTOR; TORC1, target of rapamycin complex 1; TSC, tuberous sclerosis complex; v-ATPase, vacuolar ATPase; VPS34, vacuolar protein sorting 34.

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Localizing mTOR: mechanisms via which amino acids control Rags

The search for proteins that stimulate mTORC1 in response to amino acid sufficiency resulted in the identification of

the Rag GTPases [10,11]. The GTPases RagA and RagB form constitutive heterodimers with the related RagC or RagD respectively. In the presence of amino acids, RagA/B GTPases become GTP-loaded and induce translocation of mTORC1 from the cytoplasm to lysosomes, bringing it into close proximity of Rheb [10]. The Rags do not contain a membrane-targeting sequence, but are bound to lysosomes by means of the Ragulator complex. Ragulator consists of LAMTOR [late endosomal/lysosomal adaptor, MAPK (mitogen-activated protein kinase) and mTOR activator] 1, 2 and 3 [also known as p18, p14 and MP1 (MAPK scaffold protein 1)], HBXIP (hepatitis B virus X-interacting protein) and C7orf59. Apart from binding mTORC1, Ragulator also functions as the amino acid-stimulated GEF (guanine-nucleotide-exchange factor) for RagA/B that brings these GTPases to their active GTP-bound state [12]. In order to stimulate the GEF activity of Ragulator, the lysosomal v-ATPase (vacuolar ATPase), to which it is coupled, needs to undergo a conformational change that is induced by accumulation of amino acids inside lysosomes. Supporting evidence for this inside-out mode of regulation comes from the use of v-ATPase inhibitors and overexpression of the lysosomal amino acid exporter SLC36A1 (solute carrier 36A1)/PAT1 (proton-coupled amino acid transporter 1) ([13], and see [14] for a review).

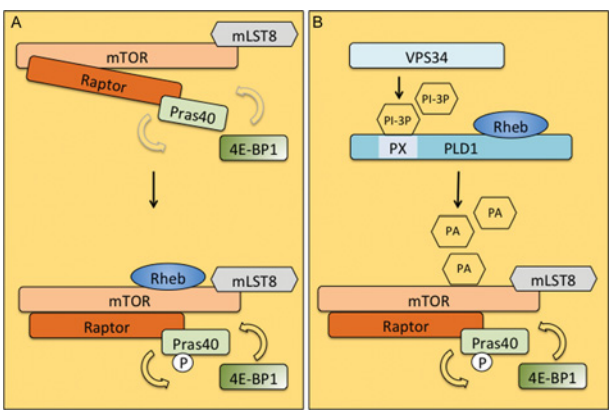
Among the amino acids that are important for mTORC1 activation, leucine is special in that it is sufficient to strongly activate mTORC1 in cells deprived of amino acids. This suggests that part of the amino-acid-sensing machinery binds leucine directly. One such molecule is LRS (leucyl-tRNA synthetase). LRS has been proposed to interact with the Rags in yeast and to keep the homologue of RagA in the GTP-bound state [15], whereas, in human cells, a role as a RagD-GAP has been postulated [16]. An alternative model for the role of leucine comes from studies on glutaminolysis. This process results in the production of high levels of 2-oxoglutarate (α-ketoglutarate) used to maintain tricarboxylic acid intermediates in mitochondria (anaplerosis) and form citrate, required for lipid production [17]. Glutaminolysis is especially important in rapidly growing cells such as tumour cells. One of the two enzymes involved in glutaminolysis, GDH (glutamate dehydrogenase) is directly bound and activated by leucine, thus resulting in enhanced 2-oxoglutarate production. Intriguingly, addition of cell-permeant 2-oxoglutarate derivatives can specifically induce lysosomal translocation and activation of mTORC1 via an increase in GTP loading of RagB, but the underlying mechanism remains to be resolved [18]. Taken together, it appears that amino acids inside lysosomes as well as cytoplasmic leucine (derivatives) contribute to amino acid sensing.

Activating mTORC1: mechanisms involving Rheb

The rapid increase in mTORC1 substrate phosphorylation following stimulation of cells with insulin or amino acids is critically dependent on Rheb. In the simplest model, GTP-bound Rheb either stimulates the kinase activity of mTORC1 via direct interaction or induces a conformational change in mTORC1 that results in enhanced substrate turnover (Figure 1A). Rheb can interact with the N-terminal part of the mTOR kinase domain in an amino-acid-dependent manner [19,20]. Surprisingly, this binding is not specific for GTP-bound Rheb and, in fact, the affinity of GDP-bound and nucleotide-free Rheb for mTOR is higher. Using *in vitro* kinase assays, Long et al. [19,20] found mTORC1 isolated from cells co-transfected with wild-type or active mutants such as Rheb^{Leu64} to be much more active than that from cells co-transfected with mutants with a decreased nucleotide affinity such as Rheb^{Asn20}. However, post-lysis addition of GTP-bound Rheb to isolated mTORC1 did not affect mTORC1's kinase activity in their hands. Also, later studies do not favour the idea that Rheb increases the catalytic activity of mTOR [21]. Enhanced substrate binding to mTORC1 is thus an attractive alternative explanation for the action of Rheb and indeed increased binding of 4E-BP1 to raptor can be detected in insulin-stimulated cells [22,23]. On the other hand, by applying a different immunoprecipitation protocol, the Sabatini laboratory demonstrated using *in vitro* kinase assays that GTP-bound Rheb can activate mTORC1 directly, whereas GDP-bound Rheb fails to do so [24]. This different procedure resulted in the maintenance of PRAS40 in mTORC1, which has an inhibitory activity that can be counteracted by Rheb-GTP. PRAS40 interacts with raptor via a so-called TOS (target of rapamycin signalling) motif that is also present in other mTORC1 substrates, leading to a model in which PRAS40 prevents access of other substrates

Figure 1 | Modes of mTORC1 activation by Rheb

(**A**) Direct mode of mTORC1 activation by Rheb depends on the interaction of Rheb with the kinase domain of mTOR. This may result in either an enhanced catalytic activity of mTOR or an increase in substrate turnover by changing the conformation of the entire complex. (**B**) Indirect mode of mTORC1 activation via PLD1. PLD1 is translocated to lysosomes via an interaction of its PX domain with PtdIns3*P* (PI-3P) produced by VPS34. Here it engages with Rheb in a GTP-dependent interaction with PLD1, which results in enhanced PA production. PA binding to mTOR enhances its activity.



such as 4E-BP1 [21,24,25]. However, given that knockdown of PRAS40 has no effect on mTORC1 in a variety of cell lines [23,26,27] and in *Drosophila* functions as a TORC1 (target of rapamycin complex 1) repressor only in oocytes [28], indicates that the picture is not yet complete (see also the discussion in [23]).

Indirect modes of mTORC1 activation by Rheb have also been described (Figure 1B) [29,30]. The most compelling evidence has been provided for PLD1 (phospholipase D1), which has all the hallmarks of an effector for Rheb: PLD1 is required for mTORC1 activation by serum or amino acids and interacts with Rheb in a GTP-dependent manner. This interaction leads to an increase in the catalytic activity of PLD1 [29]. PLD1 hydrolyses phosphatidylcholine to choline and PA (phosphatidic acid), which activates mTORC1 by binding to a region named FRB [FKBP12 (FK506-binding protein 12)–rapamycin-binding] [31]. The activity of PLD1 following serum stimulation is dependent on amino acids [29,32]. In addition, replenishing cells starved for amino acids is sufficient to stimulate PLD1. The class III PI3K (phosphoinositide 3-kinase) VPS34 (vacuolar protein sorting 34) functions upstream of PLD1 in this response [33]. VPS34, which had previously been shown to be responsive to amino acid stimulation, catalyses the formation of PtdIns3*P* [8,34]. Although there has been some debate on the importance of VPS34 (see [35] for a review), amino-acid-induced mTORC1 activity is compromised in VPS34-deficient mouse embryonic fibroblasts [36]. Remarkably, mTORC1 appears to be fully functional in these cells when grown in the presence of serum. PtdIns3*P* produced by VPS34 is a ligand for the PX (Phox homology) domain present in PLD1 and increases the catalytic activity of PLD1. In addition, PtdIns3*P* targets PLD1 to late endosome/lysosomes. Although this phospholipid is more abundant on early endosomes, it has also been detected on late endosomes/lysosomes [37]. The selective translocation of PLD1 to late endosomes/lysosomes may depend on the simultaneous interaction with GTP-bound Rheb. The VPS34/PLD1/PA pathway operates in parallel to the Rag GTPases. Indeed, when mTORC1 is constitutively targeted to lysosomes by expression of activated Rags, amino acids induce a further increase in mTORC1 activity [33].

The lysosome: the place to be for Rheb?

Activation of mTORC1 by Rheb at the lysosomal surface via amino-acid-dependent interactions of raptor with GTP-bound RagA/B provides the framework for understanding the role of amino acids. Rheb can interact directly with endomembranes by means of a C-terminal CAAX-box motif in which the cysteine residue becomes farnesylated [38]. In contrast with most other Ras-like GTPases, the C-terminus of Rheb lacks a second membrane-targeting signal such as a polybasic region or palmitoylated cysteine residues [39]. As a consequence, the association of Rheb with endomembranes is weak compared with other Ras family members. This loose association may underlie the different outcomes of immunofluorescence studies on the subcellular localization of overexpressed tagged versions of Rheb. In addition, detection of Rheb in fixed or permeabilized cells can differ from that in live-cell imaging (e.g. [40,41]). Various studies reported the presence of Rheb on the Golgi, ER (endoplasmic reticulum) and cytoplasmic vesicles ([41–43], and F.J.T. Zwartkruis and M.J. Groenewoud, unpublished work). On the basis of the use of a Golgi-targeted Rheb isoform and application of the Golgi-apparatus-disrupting drug brefeldin A, Buerger et al. [40] proposed that Rheb signals at the Golgi. However, Hanker et al. [42] demonstrated that the localization of Rheb was altered in cells lacking the enzymes for proper CAAX-box processing without affecting its capacity to stimulate mTORC1. A drawback of these studies is that the functionality of Rheb is tested in an overexpression setting. Under these conditions, Rheb becomes GTP-loaded, possibly by limiting amounts of TSC1/2, and is less dependent on its subcellular localization [44]. Lysosomal localization of Rheb was first suggested on the basis of partial co-localization with the late endosome marker Rab7 and vesicles marked by lysosome-specific compounds such as LysoTracker [45]. Rab7 also marks the compartment where mTOR is situated in the presence of amino acids, supporting the notion that Rheb activates mTOR on lysosomes [10]. Taken together, it seems unlikely that Rheb is restricted to late endosomes/lysosomes, but these organelles may well be the single membrane-limited compartment where the local concentrations of mTOR and Rheb are high enough for a functional interaction. Since overexpression results in accumulation of Rheb at the ER/Golgi as well as GTP loading, it is plausible that Rheb is activated at this site. The existence of a membrane-bound GEF is consistent with the fact that overexpressed Rheb lacking an intact CAAX-box is less GTP-bound than wild-type Rheb [46].

It is clear that the CAAX-box of Rheb is required for efficient signalling in mammalian cells [40,42], consistent with findings in yeast [47]. If the weak membrane association of Rheb does serve a special function, it remains to be seen, but various options can be envisaged. First, Rheb may leave the lysosomes in complex with mTORC1 to keep it active at sites where mTORC1 has been reported to function, such as ribosomes [48]. Alternatively, rapid association to and dissociation from lysosomes could be important for regulation of Rheb. For example, the intrinsic guanine-nucleotide-exchange rate of Rheb increases upon membrane dissociation [49], but it is unclear whether this overcomes the requirement for a Rheb-GEF. The negative regulator of Rheb, TSC1/2, is present on lysosomes [50], suggesting that dissociation from lysosomes is not necessary to terminate Rheb signalling.

The maintenance of a functional endosomal/lysosomal pathway requires adaptation of the dynamics of fusion and maturation of vesicles derived from various sources including the Golgi. Golgi vesicles will supply late endosomes and lysosomes with new hydrolases to degrade their contents, but may simultaneously target surface-located Rheb into the endosomal/lysosomal pathway [51]. This is compatible

with the observation that interfering with the maturation of early and late endosomes, e.g. by altering the expression or regulation of Rabs involved in this process, affects mTORC1 activation. Reciprocally, mTORC1 pathway components signal back to adapt the capacity of the endosomal/lysosomal system to metabolic supplies and demands. For example, mTOR may function in the maturation of lysosomes [52], whereas Rheb has been hypothesized to target the amino acid transporter PAT1 from the cell surface to lysosomes [53]. PAT1 is able to promote cell growth and activate mTORC1 [54]. Like the v-ATPase, PAT1 interacts with the Rags, and it is possible that the extralysosomal flux of amino acids via PAT1 rather than the intralysosomal concentration is the signal that impinges on the Rags. A drawback of this model, however, is that PAT1 does not transport leucine, which would be expected for an amino acid sensor. Nonetheless, mislocalization of amino acid transporters is more frequently seen in relation to mutations in the TOR (target of rapamycin) pathway. In fission yeast, loss of *tsc2* causes mislocalization of the amino acid transporter *cat1*. In budding yeast, the Rag orthologues Gtr1p and Gtr2p function in the localization of the amino acid transporter GAP1 (general amino acid permease 1) [55] and amino-acid-mediated TORC1 activation [56], indicating that many aspects of mTOR regulation are highly conserved during evolution. Phylogenetic analysis suggests that Rheb and the Rags are strongly linked during evolution (M.J. Groenewoud, T.J.P. van Dam and F.J.T. Zwartkruis, unpublished work). However, a further unravelling of the molecular mechanisms of amino acid sensing is required to understand better the spatiotemporal control of mTORC1 among various organisms.

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