

Review

## Mechanisms of amino acid sensing in mTOR signaling pathway

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### Abstract

Amino acids are fundamental nutrients for protein synthesis and cell growth (increase in cell size). Recently, many compelling evidences have shown that the level of amino acids is sensed by extra- or intra-cellular amino acids sensor(s) and regulates protein synthesis/degradation. Mammalian target of rapamycin complex 1 (mTORC1) is placed in a central position in cell growth regulation and dysregulation of mTOR signaling pathway has been implicated in many serious human diseases including cancer, diabetes, and tissue hypertrophy. Although amino acids are the most potent activator of mTORC1, how amino acids activate mTOR signaling pathway is still largely unknown. This is partly because of the diversity of amino acids themselves including structure and metabolism. In this review, current proposed amino acid sensing mechanisms to regulate mTORC1 and the evidences pro/against the proposed models are discussed.

**Key Words:** Amino acid signaling, mTOR, protein synthesis, cell growth

### mTOR signaling pathway

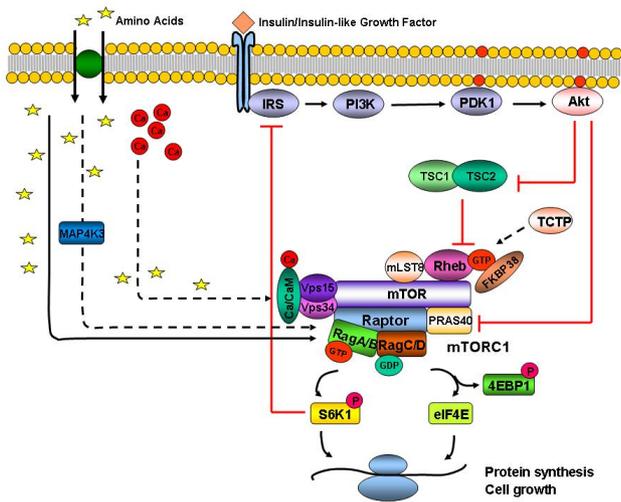
Rapamycin, an antifungal macrolide purified from *Streptomyces hygroscopicus* (Abraham & Wiederrecht, 1996) has potent immunosuppressant and antiproliferative properties and currently is used as a FDA-approved immunosuppressant and anticancer drug. Its cellular target was identified from yeast genetic screening in which mutation of TOR1-1 and TOR2-1 genes showed resistance to the growth-inhibitory properties of rapamycin (Heitman *et al.*, 1991). TOR homologs have also been identified in plants (AtTOR in *Arabidopsis thaliana*) (Menand *et al.*, 2002), fungus (TOR1 in *Cryptococcus neoformans*) (Cruz *et al.*, 1999), *Caenorhabditis elegans* (CeTOR) (Hara *et al.*, 2002), *Drosophila* (dTOR) (Oldham *et al.*, 2000; Zhang *et al.*, 2000), and mammals (mTOR) (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994). Unlike yeast TOR1 and TOR2, however, mammals have only one TOR gene.

TOR is a serine/threonine protein kinase and a member of phosphatidylinositol kinase-related kinase (PIKK) family (Schmelzle & Hall, 2000). It consists of 20 tandem HEAT repeats at the N-terminal followed by an FAT and FRB (FKBP12/rapamycin binding) domains. mTOR kinase domain is located in between FRB and FATC (FAT C-terminus) domain at the C-terminus of the protein (Inoki *et al.*, 2005a). Rapamycin binds with immunophilin FKBP12 (FK506-binding protein 12 kDa) in the cell and forms a complex (Abraham & Wiederrecht, 1996). It appears that this FKBP12-rapamycin complex binds to FRB

domain and inhibits physiological functions of mTOR, however, exact mechanism has not been elucidated yet. mTOR exists in two distinct protein complexes, mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2) (Hara *et al.*, 2002; Kim *et al.*, 2002; Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). mTORC1 consists of mTOR, Raptor, mLST8 (GβL), PRAS40 (proline-rich Akt/PKB substrate 40 kDa), and recently identified FKBP38 (Bai *et al.*, 2007; Yang & Guan, 2007). mTORC1 regulates the rate of protein synthesis and cell growth in a rapamycin sensitive way (Fig. 1) (Fingar *et al.*, 2002; Hay & Sonenberg, 2004). While in mTORC2, mTOR interacts with Rictor, mLST8, Protor (protein observed with Rictor) (Pearce *et al.*, 2007), and mSin1 (reviewed in (Yang & Guan, 2007)). Unlike mTORC1, mTORC2 activity is not inhibited by rapamycin at least in a short time period (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). Substrates of mTORC2 include Akt and SGK (serum and glucocorticoid-inducible kinase) (Garcia-Martinez & Alessi, 2008). mTORC2 also regulates maturation and stability of conventional PKC (Facchinetti *et al.*, 2008; Ikenoue *et al.*, 2008) and has known to be involved in cytoskeletal organization (Loewith *et al.*, 2002; Jacinto *et al.*, 2004). mTORC2 is activated by growth factors such as insulin but not by nutrients.

Raptor is a scaffold protein which recruits substrates to mTOR and knock-down of Raptor abolishes physiological activity of mTOR (Hara *et al.*, 2002; Kim *et al.*, 2002). mLST8 binds to mTOR kinase domain and activates the kinase activity independent of Raptor (Kim *et al.*, 2003). Recent *in vivo* studies

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**Fig. 1. Regulation of mTORC1** mTORC1 is a multiprotein complex composed of Raptor, mLST8, PRAS40, and FKBP38 and regulates protein synthesis and cell growth by phosphorylating S6K1 and 4EBP1. Rheb directly binds and activates mTOR kinase, GTP-bound active Rheb sequesters FKBP38 which otherwise binds and inhibits mTORC1. TSC1/2 heterodimer converts Rheb from GTP-bound active to GDP-bound inactive form. In *Drosophila*, TCTP was identified as Rheb GEF. mTORC1 activity is precisely coordinated by growth factors such as insulin or insulin-like growth factor and nutrients. Insulin binding to insulin receptor recruits IRS and subsequently activates PI3K and PDK1. PDK1 then phosphorylates and activates Akt which in turn phosphorylates two negative regulators of mTORC1, TSC2 and PRAS40, and relieves their inhibitory function. Amino acids stimulation does not phosphorylate Akt, however, strongly phosphorylates S6K and 4EBP1 in the absence of insulin stimulation. Recently three proteins, Rag, Vps34, and MAP4K3, have been presented as putative intracellular amino acid sensors. Each protein activates mTORC1 by distinctive mechanism. On the other hand, activated S6K1 phosphorylates and degrades IRS by negative feedback inhibition which in turn inhibits PI3K and mTORC1 signaling pathway (for details, see text). IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3 phosphate kinase; PDK1: 3-phosphoinositide-dependent protein kinase1; Note: uncertain pathway is delineated as dashed line.

using mLST8<sup>-/-</sup> MEFs (mouse embryonic fibroblasts), however, showed that an ability of mTOR to phosphorylate its substrates, S6K and 4EBP1, and to interact with Raptor was not impaired in these cells (Guertin *et al.*, 2006), which suggests that mLST8 may not be an indispensable component of mTORC1 function. PRAS40 acts as a negative regulator of mTORC1 either by binding directly to the mTOR kinase domain and inhibits kinase activity (Vander Haar *et al.*, 2007) or by association with Raptor via a TOR signaling motif (TOS motif) in PRAS40, which can cause substrate competition to Raptor (Oshiro *et al.*, 2007; Sancak *et al.*, 2007; Wang *et al.*, 2007). On the other hand, insulin stimulation phosphorylates Thr246 site of PRAS40 and relieves its inhibitory effect on mTORC1, which suggests that PRAS40 mediates growth factor signals to mTORC1. Bai *et al.* (2007) identified that FKBP38 also acts as a negative regulator of mTORC1 and overexpression of FKBP38 inhibits S6K1 (T389), S6 (S235/236), and 4EBP1 (T37/46) phosphorylation.

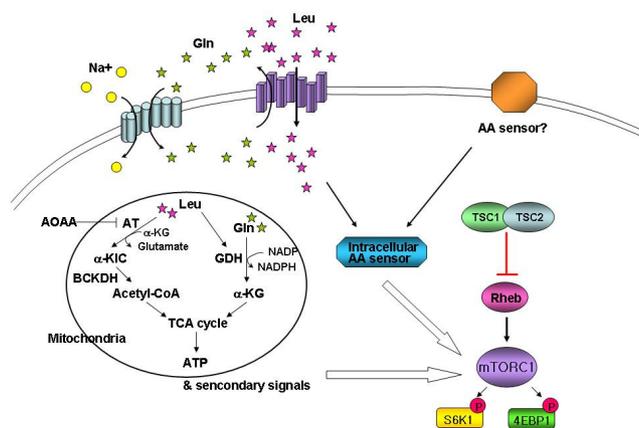
The best-characterized protein substrates of mTORC1 are S6K1 and 4EBP1. mTORC1 recruits and phosphorylates S6K1 and 4EBP1 via interaction of Raptor and TOS motif located at the N-terminus and C-terminus of S6K1 and 4EBP1, respectively (reviewed in (Fingar *et al.*, 2002; Hay & Sonenberg, 2004)).

Activated S6K1 phosphorylates 40S ribosomal protein S6, which has been suggested to selectively increase the translation of subset of mRNAs containing a terminal oligopyrimidine (TOP) tract at their 5'-end. Since many of the 5'-TOP mRNAs encode ribosomal proteins and other translation factors, activated S6K by mTOR is thought to increase ribosome biogenesis. 4EBP1 is a translational repressor. Its unphosphorylated form binds and inhibits eukaryotic translation initiation factor 4E (eIF4E) which binds to the Cap structure (m<sup>7</sup>GpppN) at the 5'-end of mRNA transcripts. Phosphorylation of 4EBP1 by mTOR dissociates tight 4EBP1 binding from eIF4E from which eIF4E recruits other components of the translation initiation complex and initiates cap-dependent translation, which increases overall translational capacity of the cell. S6K1 also phosphorylates translation inactivating kinase, eukaryotic elongation factor2 (eEF2) kinase, and inactivates its function (Redpath *et al.*, 1996). mTOR activation, therefore, promotes elongation process as well.

The activity of mTORC1 is finely tuned by upstream positive and negative regulators (Hay & Sonenberg, 2004). Rheb (Ras-homolog enriched in brain) small GTPase is a positive regulator of mTORC1 and is indispensable for mTOR activity. It directly binds to the carboxyterminal lobe of the mTOR catalytic domain and modulates the kinase activity (Long *et al.*, 2005a; Sancak *et al.*, 2007). S6K1 and 4EBP1 can not be phosphorylated if Rheb gene is knocked down (Zhang *et al.*, 2003), whereas, overexpression of Rheb stimulates S6K1 and 4EBP1 phosphorylation (Inoki *et al.*, 2003a; Tee *et al.*, 2003). TSC (tuberous sclerosis complex) 1 and 2 heterodimer acts as Rheb GAP (GTPase activation protein) and converts Rheb to GDP-bound inactive form (Garami *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003). TSC1/2 integrates a variety of signals including insulin, growth factors, and energy status and negatively regulates mTORC1 (Inoki *et al.*, 2003b). Recently, *Drosophila* TCTP (translationally controlled tumour protein) has been reported as dRheb GEF (GTPase exchange factor) and activates dTOR (Hsu *et al.*, 2007). Whether mammalian TCTP also can act as Rheb GEF or not needs to be examined.

### Mechanisms of amino acid sensing

The first indication that amino acids are involved in mTORC1 pathway was shown in the study of hepatocyte autophagy (Blommaert *et al.*, 1995) in which cellular organelles are encompassed by intracellular double membrane followed by formation of autophagosome-lysosome complex and degraded (Shitani & Klionsky, 2004). In the study, addition of amino acids inhibited valine release as a result of autophagic protein degradation in the cell and this inhibition was sharply correlated with the increase of S6 phosphorylation. Moreover, the effects of the amino acids were inhibited by rapamycin. Then the question is how the level of amino acids is sensed and signals to mTORC1 (Fig. 2).



**Fig. 2.** Current proposed mechanisms of amino acid sensing by mTORC1. Amino acids are imported into the cells via various amino acid transporters and activate mTORC1 either by amino acid metabolites or by undefined membrane/intracellular amino acid sensor(s). Figure depicts a case of L-leucine (for details, see text). AT: aminotransferase;  $\alpha$ -KIC:  $\alpha$ -ketoisocaproic acid; BCKDH: branched-chain  $\alpha$ -ketoacid dehydrogenase; GDH: glutamate dehydrogenase; AOAA: aminooxyacetic acid

#### Amino acid sensing on the cell membrane

Amino acids are imported to the cells via various amino acid transporters on the cell membrane. Miotto *et al.* (1992; 1994) tested the hypothesis whether amino acid signaling is initiated from the membrane transporter using nontransportable peptide Leu<sub>8</sub>.MAP which contains eight leucine (the single most effective amino acid) residues outside and a leucine analogue, Isovaleryl-L-Carnitine (IVC). Treatment of isolated rat hepatocytes with these compounds inhibited macroautophagy and proteolysis, which indicates that Leu<sub>8</sub>.MAP, IVC, and probably free leucine must be recognized at the plasma membrane or within an associated vacuole. Different group, however, reported that only after over 1 hour treatment of the Leu<sub>8</sub>.MAP could phosphorylate S6 in some degree and moreover, in rat adipocytes, S6K1 and 4EBP1 phosphorylation was unresponsive to Leu<sub>8</sub>.MAP or IVC (Lynch *et al.*, 2000). Another concern on this hypothesis is that amino acid transporter systems have low specificity or strictness in the kind of amino acids or in structural requirement (Hyde *et al.*, 2003). For example, system L amino acid transporter can import isoleucine, valine, methionine, phenylalanine, and histidine to the cells other than leucine. Conversely, pretreatment of 2-amino-3-norbornane carboxylic acid (BCH), a selective system L amino acid transporter inhibitor, did not inhibit leucine-induced mTORC1 activity because leucine could be imported through other transporter system (Lynch *et al.*, 2000). On the other hand, it has been suggested that amino acids-induced cell swelling is a mechanism of amino acids-mediated mTORC1 activation. Some amino acids are transported into the cell via Na<sup>+</sup>-dependent transporters from which intracellular osmolarity is increased due to imported Na<sup>+</sup> and accumulated amino acid metabolites (Hyde *et al.*, 2003). Cell swelling itself has shown to exert similar effects as insulin in which protein synthesis is stimulated and protein degradation is inhibited (reviewed in (Haussinger, 1996)).

So far, however, how cell swelling regulates these processes is not known. Moreover, the most effective leucine transporter, system L amino acid transporter, is Na<sup>+</sup>-independent. This suggests that intracellular amino acid concentration or intracellular receptor, but not a particular membrane amino acid transporter, is responsible for the regulation of mTORC1 signaling pathway.

#### Amino acid sensing by aminoacyl tRNA formation

Bacteria and yeast recognize amino acids starvation by increased concentration of deacylated tRNA or reduced availability of aminoacylated tRNA. In Jurkat cells, amino acid alcohols which compete with amino acids prevent aminoacyl-tRNA formation and inhibit S6K1 phosphorylation (Iiboshi *et al.*, 1999). Inhibition of protein synthesis by applying translation inhibitors such as cycloheximide (CHX) and puromycin has shown to increase S6K1 phosphorylation in the amino acids starvation condition (Iiboshi *et al.*, 1999). These data suggest that increased level of intracellular amino acids resulted from translation inhibition and of aminoacylated tRNA is responsible for the amino acid sensing in mammalian cells as well. However, this is not consistent with time frame in which mTORC1 is activated. The level of intracellular amino acids is not changed during short time (30 min) amino acids starvation or stimulation, whereas, S6K1 phosphorylation is changed within this short time frame. Moreover, in rat adipocytes, leucine alcohol (leucinol) treatment did not affect 4EBP1 phosphorylation (Lynch *et al.*, 2000). On the other hand, it appears that Rheb and TOR are not involved in nutrient import. Suppression of Rheb and TOR gene expression by RNAi in *Drosophila* S2 cells did not affect nutrients (glucose & bulk amino acids) import but significantly reduced ribosome biogenesis, protein synthesis, and cell size (Hall *et al.*, 2007). Taken together, these data raise a question on this hypothetical mechanism as well.

#### Amino acid sensing by amino acid metabolites

Imported amino acids are subjected for amino acid metabolism and there are several reports suggesting that amino acid metabolites including ATP are responsible for mTORC1 activation. For example, leucine is metabolized to  $\alpha$ -ketoisocaproic acid ( $\alpha$ -KIC) by reversible aminotransferase (AT), which is then further metabolized to TCA cycle intermediates by branched-chain  $\alpha$ -ketoacid dehydrogenase (BCKDH). Otherwise, leucine as it is can be an allosteric activator of glutamate metabolizing enzyme, glutamate dehydrogenase (GDH) (Fahien *et al.*, 1988; Sener & Malaisse, 1980). The consequence of leucine metabolic pathways would be production of ATP and other metabolically-linked secondary signals, which was proposed as a mechanism of mTOR signaling pathway activation by leucine in pancreatic  $\beta$ -cells (McDaniel *et al.*, 2002). In fact, Dennis *et al.* (2001) have previously reported that mTOR is an intracellular ATP

sensor and ATP depletion caused by incubation of cells with 2-deoxyglucose or several mitochondria inhibitors including rotenone and antimycin A inhibits S6K1 and 4EBP1 phosphorylation (Inoki *et al.*, 2003b; Xu *et al.*, 2001). A concern on this hypothesis is that the concentration of ATP is not affected by short time amino acids deprivation or stimulation in most mammalian cells (Dennis *et al.*, 2001). Nevertheless, it has been shown that an AT inhibitor, aminooxyacetic acid (AOAA), inhibits S6K1 phosphorylation upon leucine treatment in pancreatic  $\beta$ -cells (Xu *et al.*, 2001). A nonmetabolized leucine analog,  $\beta$  ( $\pm$ ) 2-aminobicyclo [2.2.1] heptane-2-carboxylic acid, also failed to stimulate S6K1 phosphorylation in the absence of leucine (McDaniel *et al.*, 2002). Moreover, proteolytic dose responses and inhibition of autophagy to L-leucine and to IVC, readily metabolizable leucine analogue, were similar in the perfused rat liver (Miotto *et al.*, 1992). Stimulation with  $\alpha$ -KIC phosphorylates 4EBP1 in the absence of leucine in adipocytes (Lynch *et al.*, 2000). Taken together, this suggests that although it is difficult to pinpoint exactly what it is, certain products of leucine oxidation are responsible for mTORC1 activation.

In the liver, however, leucine is poorly metabolized owing to its low rate of transamination. Therefore, we still can not exclude a possibility that the effect of leucine on the mTORC1 activation is due to leucine itself at some undefined locus of recognition. Leucine metabolizing enzyme, AT, is a reversible aminotransferase which also can convert  $\alpha$ -KIC to leucine. Interestingly,  $\alpha$ -KIC induced phosphorylation of 4EBP1 was less than leucine induced 4EBP1 phosphorylation and moreover,  $\alpha$ -KIC effect was attenuated in the presence of transaminase inhibitor, L-cycloserine (Lynch *et al.*, 2000). Lynch *et al.* (2000) also found that norleucine which is not incorporated in protein translation was as effective as leucine in 4EBP1 phosphorylation, which suggests that there may be a specific intracellular leucine receptor, which recognizes the specific structure of leucine. However, leucine analogues which contain either strong leucine agonist or antagonist activity in one cell line do not always exert the same effect in different cell lines. Therefore, this proposed mechanism can be cell-type specific.

#### *Amino acid sensing by mTORC1*

Although the mechanism is not known, it is obvious that amino acids regulate mTORC1 activity (Hara *et al.*, 1998). That is, amino acids stimulation increases S6K1 and 4EBP1 phosphorylation, whereas, amino acids deprivation rapidly dephosphorylates S6K1 and 4EBP1. Therefore, extensive research efforts have centered on mTORC1 regulation itself.

Ectopic overexpression of Rheb completely inhibited S6K1 and 4EBP1 dephosphorylation upon amino acids withdrawal (Long *et al.*, 2005a; 2005b; Saucedo *et al.*, 2003). Since Rheb activates mTORC1 activity by directly binding to mTOR, Rheb-mTOR interaction was examined. Long *et al.* (2005b) showed that Rheb-mTOR interaction was in fact reduced upon amino acids

withdrawal, whereas the interaction was fully recovered when amino acids alone were added to D-PBS. Amino acids, however, did not change Rheb-Raptor and Rheb-mLST8 interaction (Long *et al.*, 2005b). Curiously, however, Rheb-mTOR interaction is not affected by nucleotide binding status of Rheb (Long *et al.*, 2005a). The binding of nucleotide-free mutant of Rheb to mTOR was even stronger than that of wild-type Rheb, although it impaired mTOR kinase activity. Amino acids modulation of nucleotide binding status of Rheb is also not in consistency. For example, GTP level of overexpressing Rheb was increased by amino acids stimulation (Smith *et al.*, 2005), but endogenous Rheb-GTP charging was not changed by amino acids withdrawal (Nobukuni *et al.*, 2005). Roccio *et al.* (2006) reported that amino acids starvation did not change Rheb-GTP level, but add back amino acids clearly increased Rheb-GTP loading. In consistent with this, TSC1/2 is also not required for amino acids-mediated mTORC1 regulation (Roccio *et al.*, 2006; Smith *et al.*, 2005). Amino acids withdrawal still dephosphorylated S6K1 and 4EBP1 independent of TSC2 gene expression (Nobukuni *et al.*, 2005; Smith *et al.*, 2005). On the other hand, it has been proposed that amino acids regulate mTORC1 activity by modulating mTOR-Raptor interaction. Amino acids starvation causes tighter binding of Raptor to mTOR which inhibits mTOR kinase activity whereas amino acids stimulation loosens the interaction (Kim *et al.*, 2002). The mLST8-mTOR interaction was not affected by amino acids. This suggests that amino acids specifically modulate mTOR-Raptor interaction and regulate mTOR kinase activity.

#### *Amino acid sensing by intracellular proteins*

Recently, several intracellular proteins have been identified as intracellular amino acid sensors which include hVps34, MAP4K3, and Rag.

**Vps34:** Vps34 is a Class III PI3K which forms an active complex with another kinase Vps15 and generates PtdIns(3)P by phosphorylating the 3' hydroxyl position of the phosphatidylinositol ring (reviewed in (Backer, 2008; Nobukuni *et al.*, 2007; Yan & Backer, 2007)). Vps34 was initially known to play an important role in vesicular trafficking (Lindmo & Stenmark, 2006; Odorizzi *et al.*, 2000) and a role of Vps34 in amino acids sensing has been recently reported (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005). That is, hVps34 activity as well as PtdIns(3)P production was increased by amino acids stimulation. On the contrary, suppression of hVps34 gene expression with hVps34 siRNA or overexpression of GFP-FYVE (Fab1p, YOTB, Vac1p, EEA1 (early endosomal antigen 1)) expression vector which acts as a dominant negative by sequestering PtdIns(3)P impaired S6K1 and 4EBP1 phosphorylation but did not affect insulin-stimulated Akt phosphorylation. It appears that, however, amino acids do not directly regulate hVps34 activity. Instead, amino acids stimulation facilitates  $\text{Ca}^{2+}$  influx, which in turn increases the interaction of  $\text{Ca}^{2+}$ /CaM with the hVps34-mTOR

and activates mTORC1 (Gulati *et al.*, 2008). There are several concerns, however, on presenting hVps34 as an amino acids sensing system to activate mTORC1. Ectopic expression of hVps34 to mammalian cells increased S6K phosphorylation only in the presence, but not in the absence of amino acids (Nobukuni *et al.*, 2005). In the study of Yan *et al.* (2009) hVps34 activity was not affected by  $\text{Ca}^{2+}$  chelators or CaM inhibitors. Most of all, in a recent *Drosophila in vivo* study, loss-of-function mutant animals of *Drosophila* Vps34 showed disrupted autophagy and endocytosis but TOR signaling was not affected by disruption of Vps34 gene (Juhasz *et al.*, 2008). That is, cell size was similar between Vps34 mutant and wild-type cells and moreover, modulation of cell size by overexpressing Rheb (increased cell size) or TSC1/2 (decreased cell size) was not affected by Vps34 mutation. Nevertheless, amino acids starvation induced autophagy in control animals but not in Vps34 mutant animals. Considering that reported function of hVps34 in amino acid signaling has been all performed in cell culture system, this observation *in vivo* raises a significant question on the proposed model.

**MAP4K3:** Findlay *et al.* (2007) identified MAP4K3 protein kinase as an important intracellular protein in amino acids sensing. They showed MAP4K3 kinase activity was regulated by amino acids and overexpression of MAP4K3 specifically increases S6K phosphorylation even in the amino acids starvation condition in a TSC1/2 independent manner. This is a difference between hVps34 and MAP4K3 in amino acids sensing since ectopic expression of hVps34 increases S6K1 phosphorylation only in the presence of amino acids. Nevertheless, it would be interesting to address a question whether overexpression of MAP4K3 increases PtdIns(3)P either in the presence or absence of amino acids.

**Rag:** Most recently, two independent research groups newly discovered that Rag (Ras-related GTPases) small GTPases (RagA, B, C, and D) play an important role in amino acid signaling (Kim *et al.*, 2008; Kim & Guan, 2009; Sancak *et al.*, 2008).

In *Drosophila* S2 cells, knock-down of dRag gene expression impaired dS6K phosphorylation upon amino acids stimulation (Kim *et al.*, 2008). In mammalian cells, expression of GTP-bound active RagA<sup>QL</sup>/RagB<sup>QL</sup> overcomes S6K1 dephosphorylation in amino acids deprivation condition, whereas GDP-bound inactive RagA<sup>TN</sup>/B<sup>TN</sup> inhibited S6K1 phosphorylation upon amino acids stimulation. Moreover, dRagA<sup>QL</sup> expressing cells showed bigger cell size than neighboring wild type cells and inhibited starvation-induced autophagy in *Drosophila*. Sancak *et al.* (2008) identified RagC as a Raptor interacting protein using a purification method. Amino acids increase GTP-level of RagB and RagB<sup>QL</sup>-RagC<sup>SN</sup> heterodimer increases mTORC1 activity by increasing Raptor-mTOR interaction. On the other hand, in RagB<sup>QL</sup> expressing cells, mTOR was already present on the Rab7 positive peri-nuclear and vesicular structures even in the amino acids deprivation condition. Interestingly, hVps34 and hVps15

also co-localize to Rab7-positive late endosome. This suggests that the function of Rag, mTOR, and hVps34 can be interconnected on Rab7 positive endosome membrane and may involve in amino acids transporter trafficking to the cell membrane as yeast Rag homologue, Gtr does (Gao *et al.*, 2006).

Recently, Nicklin *et al.* (2009) found that L-glutamine uptake by solute carrier family1 (SLC1A5) transporter is important for L-leucine/essential amino acids (EAA) import into the cells. Simultaneous efflux of L-glutamine out of cells through SLC7A5/SLC3A2, a bidirectional transporter, facilitates L-leucine/EAA import into the cells from which mTORC1 is activated. In contrast to L-glutamine, L-glutamic acid and  $\alpha$ -ketoglutarate pretreatment could not activate S6K1 upon EAA treatment, which suggests that L-glutamine effect on S6K1 is not due to increasing cellular energy level. Interestingly, previously performed large-scale genetic array analysis identified that protein mutations involved in glutamate/glutamine homeostasis also genetically interact with Gtr2 (Dubouloz *et al.*, 2005). Lethal phenotype of these mutants was reversed either by glutamate supplement in the medium or by introduction of Gtr2-expression plasmids. Moreover, Gtr2 mutant suppressors identified from genome-wide screen include genes which are directly or indirectly involved in glutamate/glutamine synthesis/degradation. It has been shown that Gse complex consisted of five proteins encoded by Gtr1, Gtr2, YBR077C (EGO3), YKR007W (EGO1), and LTV1 genes plays an important role in recycling of general amino acid permease, Gap1p, from the late endosome to the plasma membrane in yeast (Gao *et al.*, 2006). Therefore, it will be interesting to address a question whether Rag affect SLC1A5 and SLC7A5/SLC3A2 transporter trafficking and whether imported L-leucine/EAA activates mTORC1 through Rag.

## Concluding remarks

How amino acids regulate protein synthesis and cell growth has been a fundamental biological question in the field. Since inhibition of TOR pathway mimics amino acids starvation condition and moreover, amino acids are the most potent activator of TOR, extensive research efforts have centered on elucidating a mechanism of amino acids sensing in mTOR signaling pathway. From the cell membrane to intracellular proteins, each path of amino acids has been examined for a possible amino acid sensing point, however, there have been still many controversies in experimental data due to such a structural and metabolic diversity of amino acids. Recent identification of hVps34, MAP4K3, and Rag GTPases as important intracellular proteins in amino acid signaling and of a mechanism of L-Leucine/EAA import into the cells made a significant progress in our understanding of amino acids-mediated mTOR activation. However, how imported amino acids are sensed by hVps34, MAP4K3, or Rag GTPases and activate mTORC1 still remains as an open question. The importance of understanding amino acid signaling is that it has

many significant physiological and pathological implications *in vivo*. Anabolic response of the body after protein meal ingestion is closely correlated with phosphorylation of S6K1 and 4EBP1 in mice, rat, and human (Balage *et al.*, 2001; Long *et al.*, 2000; Shah *et al.*, 2000). Insulin production in pancreatic  $\beta$ -cells is increased by amino acids stimulation (May & Buse, 1989) and amino acids infusion increases the sensitivity of muscle protein synthesis to insulin (Garlick & Grant, 1988). Furthermore, activated S6K in turn phosphorylates and degrades insulin receptor substrate (IRS) and inhibits PI3K signaling pathway as a negative feedback inhibition, which can decrease insulin sensitivity and lead to diabetes (Fig. 1). Overactivated mTORC1 signaling pathway is frequently found in various cancers and tissue/organ hypertrophy as well (Guertin & Sabatini, 2007; Inoki *et al.*, 2005b; Lee *et al.*, 2007). Although rapamycin has been used as an FDA-approved drug to treat some of the cancers including sarcoma, suppression of S6K phosphorylation can result in activating PI3K signaling which can further exacerbate the disease state. Therefore, if we elucidate a mechanism on the amino acid signaling pathway to mTORC1 activation, more specific and effective way to prevent or treat these diseases can be developed.

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