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Commentary

A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling

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Abstract

Originally discovered as an anti-fungal agent, the bacterial macrolide rapamycin is a potent immunosuppressant and a promising anti-cancer drug. In complex with its cellular receptor, the FK506-binding protein (FKBP12), rapamycin binds and inhibits the function of the mammalian target of rapamycin (mTOR). By mediating amino acid sufficiency, mTOR governs signaling to translational regulation and other cellular functions by converging with the phosphatidylinositol 3-kinase (PI3K) pathway on downstream effectors. Whether mTOR receives mitogenic signals in addition to nutrient-sensing has been an unresolved issue, and the mechanism of action of rapamycin remained unknown. Our recent findings have revealed a novel link between mitogenic signals and mTOR via the lipid second messenger phosphatidic acid (PA), and suggested a role for mTOR in the integration of nutrient and mitogen signals. A molecular mechanism for rapamycin inhibition of mTOR signaling is proposed, in which a putative interaction between PA and mTOR is abolished by rapamycin binding. Collective evidence further implicates the regulation of the rapamycin-sensitive signaling circuitry by phospholipase D, and potentially by other upstream regulators such as the conventional protein kinase C, the Rho and ARF families of small G proteins, and calcium ions. As the mTOR pathway has been demonstrated to be an important anti-cancer target, the identification of new components and novel regulatory modes in mTOR signaling will facilitate the future development of diagnostic and therapeutic strategies.

Keywords: Rapamycin; Mammalian target of rapamycin (mTOR); Phosphatidylinositol 3-kinase (PI3K); Phosphatidic acid; Phospholipase D (PLD)

1. Introduction

The bacterial macrolide rapamycin (also known as rapamune or sirolimus), initially isolated as an anti-fungal agent, has elicited tremendous medical interest due to its multitude of activities. As an immunosuppressant, rapamycin is used to prevent graft rejection after transplantation. A recent clinical trial has demonstrated great efficacy of rapamycin-eluting stents in preventing restenosis [1]. Furthermore, rapamycin and its analogue CCI-779 are promising anti-cancer drugs, potently inhibiting a wide range of human tumor cell lines and xenografts [2,3]. All these clinically relevant activities derive from the inhibitory action of rapamycin on cell growth and proliferation. Complexed with its cellular receptor, FKBP12, rapamycin binds and inhibits the protein TOR, which is conserved in yeast [4–6], flies [7,8], worms, plants, and mammals [9–12]. The TOR proteins belong to the family of phosphatidylinositol kinase-like kinases [13], which is a unique family of large proteins (250- to 450-kDa single polypeptides) with Ser/Thr kinase activities. Tor1p and Tor2p in *Saccharomyces cerevisae* control a wide range of growth-related cellular functions, including transcription, translation, and reorganization of the actin cytoskeleton (reviewed in Ref. [14]). mTOR (also named FRAP or RAFT1) [9–12] likely plays similar pleiotropic and essential roles in regulating mammalian cellular functions, although some of the biochemical pathways may be distinct from those in yeast.

The best known function of mTOR, in the context of cell proliferation, is the regulation of translation initiation

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Abbreviations: FKBP12, FK506-binding protein; TOR, target of rapamycin; mTOR, mammalian TOR; PI3K, phosphatidylinositol 3-kinase; PA, phosphatidic acid; PLD, phospholipase D; PKC, protein kinase C; cPKC, conventional PKC; S6K1, ribosomal subunit S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ERK, extracellular signal regulated kinase; FRB, FKBP12-rapamycin binding; LPA, lysophosphatidic acid; GPCR, G protein coupled receptor; DAG, diacylglycerol; DGK, DAG kinase; PTEN, phosphatase and tensin homologue deleted from chromosome 10; LPAAT, lysophosphatidic acid acyltransferase.

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(reviewed in Ref. [15]), presumably mediated by S6K1 and 4E-BP1. S6K1 phosphorylates S6, which leads to enhanced translation initiation of the 5′ terminal oligopyrimidine tract-containing mRNAs encoding components of the protein synthesis machinery (ribosomal proteins and elongation factors) [16–19]. 4E-BP1 binds and inhibits the function of eIF4E, which assembles the 5′ CAP-binding complex for translation initiation of mRNAs containing secondary structures in their 5′ untranslated regions (reviewed in Refs. [15,20]). Mitogenic stimulation leads to phosphorylation of both S6K1 and 4E-BP1, activating the former and inactivating the latter, and thus initiating translation. mTOR is required for the mitogenic response of these proteins, as rapamycin completely inhibits the activation of S6K1 and the phosphorylation of 4E-BP1. The kinase activity of mTOR is necessary, but not sufficient, for this regulation [21,22]. The molecular mechanisms of mTOR signaling and rapamycin action have attracted major research efforts, and significant advances have been made in recent years. In this review, we discuss the recent discovery of a new pathway in mTOR signaling, and its implication in a rapamycin inhibitory mechanism and other putative components in the rapamycin-sensitive signaling circuitry.

2. Phosphatidic acid-mediated mTOR signaling: a novel mitogenic link

2.1. Two upstream signals required for mTOR downstream signaling

Two types of upstream signals are required for S6K1 activation and 4E-BP1 phosphorylation: one is mitogen-based while the other is dependent upon amino acid availability. While PI3K is essential in relaying mitogenic signals to S6K1/4E-BP1 via the Ser/Thr kinases Akt, PDK1, and atypical PKCs (reviewed in Refs. [15,19]), mTOR appears to sense the availability of amino acids [23–25] as well as cellular ATP levels [26], although exactly how amino acid levels are sensed is still unknown. An issue of debate has been whether mTOR also receives mitogenic signals. A modest increase of mTOR catalytic activity upon mitogenic stimulation has been reported [27,28], and it has been proposed that mTOR may lie downstream of PI3K and Akt [29,30]. Ser^{2448} in mTOR has been identified as a site phosphorylated by Akt *in vivo*, and the phosphorylation is indeed dependent upon PI3K [29,30]. However, it is not clear what role this phosphorylation plays in mTOR regulation since mTOR carrying an alanine mutation at this site is fully capable of activating S6K1 *in vivo* [30]. Furthermore, a rapamycin-resistant and wortmannin-sensitive S6K1 mutant (with the N-terminal 23 a.a. and C-terminal 104 a.a. deleted; [31]) is resistant to amino acid withdrawal [23], thus separating the amino acid signal and mTOR from PI3K signaling. Taken together, the amino acid-sensing mTOR pathway and the mitogenic PI3K pathway appear to be parallel, and both are required for S6K1 activation.

2.2. PA as a link between mitogens and mTOR signaling

Most recently, the lipid second messenger PA has been revealed to be a critical component of the mTOR pathway [32]. PA is speculated to participate in a variety of intracellular signaling events, and it can function as a mitogen in some cell types (reviewed in Ref. [33]). The normally low concentrations of PA in cellular membranes increase as a result of mitogenic actions of various growth factors and hormones, most likely through activation of PLD (reviewed in Refs. [33,34]). A rapid increase of PA levels upon serum stimulation accompanies the activation of mTOR signaling in human embryonic kidney (HEK) 293 cells [32]. The increase of PA is abolished by a low concentration of 1-butanol, a primary alcohol that blocks PLD-catalyzed conversion of phosphatidylcholine (PC) to PA by forming phosphatidylalcohol [35]. In the same cells, 1-butanol specifically inhibits serum-stimulated S6K1 activation and 4E-BP1 phosphorylation, but not the activation of MAP kinase (ERK1/ERK2) or Akt [32]. Similarly, 1-butanol inhibits LPA-activated S6K1 in Swiss3T3 cells [36]. A homologue of S6K1, S6K2, is also inhibited by 1-butanol [37]. Therefore, PA may be a mediator of mitogenic activation of mTOR signaling to all relevant downstream effectors. The notion that PA may be a critical component of the mTOR pathway is further supported by the finding that exogenous PA stimulates S6K1 activation and 4E-BP1 phosphorylation in the absence of mitogens [32]. In addition, serum activation of a rapamycin-insensitive/wortmannin-sensitive S6K1 mutant is resistant to butanol treatment [32], confirming that PA transduces the signal through mTOR and not PI3K.

2.3. Three pathways required in parallel for mTOR signaling to S6K1/4E-BP1

The ability of PA to stimulate S6K1/4E-BP1 is completely dependent upon amino acid sufficiency in the cell [32]. Thus, PA may govern signaling parallel to the amino acid-sensing pathway, which is consistent with PA being on a mitogenic pathway. Although PA can mimic mitogens in the activation of mTOR downstream signaling, PI3K is not activated in PA-treated cells [32]. Nonetheless, wortmannin blocks the action of PA, suggesting that the basal activity of PI3K is required for PA to stimulate S6K1 activation and 4E-BP1 phosphorylation. The simplest model explaining these observations is one in which three pathways converge on S6K1/4E-BP1 (Fig. 1): an amino acid-sensing mTOR pathway, a mitogen-activated PI3K pathway, and a mitogen-activated PA-mTOR pathway (likely mediated by PLD). In this model, mTOR integrates mitogenic and nutrient signals as the master regulator.

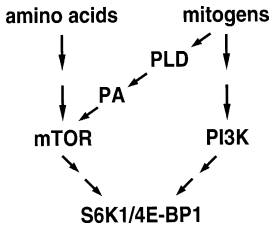

Fig. 1. Convergence of three pathways to regulate S6K1 and 4E-BP1.

3. Direct interaction between PA and the FRB domain in mTOR: a potential mechanism of rapamycin action

3.1. In vitro interaction between PA and FRB

The FRB domain in mTOR has been mapped to amino acids 2025 to 2114, located immediately N-terminal to the sequence-defined kinase domain [12,38]. A regulatory role has been speculated for the FRB domain as it is the direct target site of rapamycin, and also because a drastic dominant negative effect on progression through the G1 phase of the cell cycle was observed when purified FRB protein was microinjected into human osteosarcoma MG63 cells [39]. In search of a putative regulator interacting with the FRB domain, we have considered lipid molecules as candidates because the crystal structure of the FRB domain reveals a four-helical bundle [40], reminiscent of many apolipoproteins [41]. Indeed, the FRB protein binds PA-containing small unilamellar vesicles with strikingly high selectivity [32]. Preincubation with the rapamycin–FKBP12 complex abolishes the ability of FRB to bind PA, consistent with the possibility that rapamycin may compete with PA for binding to the FRB domain.

Similar to other PA-binding proteins, an electrostatic interaction appears important for FRB binding to PA, as high ionic strength partially disrupts the binding [32]. A group of basic residues that are present at the FRB–rapamycin interface [40] has been examined, and Arg^{2109} has been found to be important for PA binding, as the Arg^{2109}Ala mutant FRB displays 50–60% of the wild-type affinity for PA [32]. The extent of loss of affinity by this mutation is comparable to that observed in the presence of high ionic strength. The remaining affinity is likely due to hydrophobic interactions between PA and the protein. A direct interaction has also been observed *in vitro* between the full-length mTOR and a biotinylated PA (Fang Y, Qian L, Prestwich G, and Chen J, unpublished observation). However, an *in vivo* interaction between PA and mTOR has not been demonstrated due to technical limitations.

3.2. Correlation between PA–FRB interaction and mTOR signaling

When introduced into full-length mTOR, the Arg^{2109}Ala mutation diminishes signaling to S6K1 by about 40% [32]. The close correlation between PA–FRB binding and mTOR signaling fully supports the critical role of PA in the mTOR pathway. mTOR may thus be added to the growing list of signaling proteins proposed to be regulated by PA, including some protein kinases, protein phosphatases, a lipid kinase, and a phospholipase (reviewed in Ref. [33]).

A large fraction of cellular mTOR appears to associate with intracellular membranes ([42–44] and unpublished observations). The discovery of the association of PA with mTOR has led to the speculation that PA may serve to anchor mTOR in the membrane. This possibility, however, has been ruled out by the observation that a mutant mTOR with the FRB domain deleted associates with cellular membranes to the same extent as the wild-type protein (unpublished observations). In fact, several segments within the N-terminal two-thirds of mTOR independently target mTOR to membranes, similar to the observation that putative HEAT motifs in the N-terminal region of Tor2p are capable of anchoring the protein in yeast cell membranes [45]. Hence, it appears that PA may regulate mTOR signaling by a mechanism other than controlling the membrane association of mTOR.

3.3. A putative mechanism of rapamycin's action

The mechanism for rapamycin inhibition has been a major issue of debate. An obvious possibility is that rapamycin inhibits the catalytic activity of mTOR, and the rapamycin–FKBP12 complex indeed partially blocks purified mTOR kinase activity *in vitro*. However, *in vitro* inhibition requires much higher rapamycin concentrations than what is effective *in vivo* (see, for example, Ref. [21]), and mTOR bound to immobilized rapamycin–GST–FKBP12 is fully active compared to the unbound protein (unpublished observation). In addition, the yeast Tor2p has an essential role in regulating actin cytoskeleton reorganization [46], which is dependent upon the kinase activity of Tor2p, but insensitive to rapamycin, suggesting the separation of TOR kinase activity and rapamycin action [47]. Finally, the kinase activity of mTOR is also separated from rapamycin inhibition in skeletal myogenesis, where a rapamycin-sensitive but kinase-independent mechanism seems in action [48].

PA and rapamycin compete with each other for binding to FRB, suggesting that rapamycin inhibition may be a result of displacing PA from mTOR *in vivo*. What would be the molecular consequence of PA–mTOR interaction? We have observed that neither PA nor butanol has any effect on the kinase activity of mTOR *in vitro* or *in vivo* (unpublished data). In addition, mTOR mutants that have diminished affinity for PA display wild-type kinase activity [32]. Thus, PA does not appear to regulate mTOR catalytic activity, which is consistent with the notion that rapamycin does not inhibit mTOR kinase activity. It is not clear exactly how PA regulates the signaling activity of mTOR, but several

possibilities exist. For instance, PA may help recruit a downstream effector, remove a negative regulator, or modulate mTOR kinase specificity toward a physiological substrate. In any case, the mutually exclusive interactions of FRB–PA and FRB–rapamycin suggest that rapamycin likely exerts its inhibitory effect by blocking PA binding to mTOR *in vivo*. It remains to be seen whether this role of PA is specific for mammals or universal in eukaryotes.

4. Potential upstream regulators of mTOR

4.1. PLD

The emergence of PA as an activator of mTOR, together with butanol's inhibitory effect, suggests a critical involvement of PLD in the mTOR pathway. Two genes have been cloned in mammals that encode PLD activities: *PLD1* and *PLD2*. While PLD2 has a high basal activity in most mammalian cells, PLD1 is known to respond to various mitogens and agonists and is regulated by the ARF and Rho families of small G proteins as well as the cPKC (reviewed in Refs. [34,49,50]). Thus, PLD1 is the likely candidate to mediate mitogenic stimulation of PA upstream of mTOR. When co-expressed with S6K1, a catalytically inactive PLD1 [51] acts as a dominant negative mutant by inhibiting the mitogenic stimulation of S6K1 activity (unpublished observation), providing further evidence that PLD1 may indeed be an upstream component of the mTOR pathway. Definitive proof of the role of PLD in mTOR signaling awaits targeted gene disruption or RNA interference experiments, as specific inhibitors of PLD are not available.

Many agonists for GPCRs, as well as growth factors and cytokines, activate PLD [34]. S6K1 activation has been observed to result from signaling downstream of both GPCRs and other types of receptors such as receptor tyrosine kinases [52]. An intriguing question is whether PLD is involved in various types of receptor signaling to mTOR, and, if so, whether distinct regulators mediate PLD activation downstream of different receptors.

4.2. Rho

Three G proteins in the Rho family, RhoA, Cdc42, and Rac1, have all been found to directly interact with and activate PLD1 (reviewed in Refs. [34,49]). Interestingly, constitutively active Cdc42 and Rac1 have been shown to activate S6K1 *in vivo* [53]. Although the activated Cdc42 and Rac1 interact with S6K1, the interaction is not sufficient for activation of S6K1 [53]. It has thus been proposed that the membrane recruitment of S6K1 via Cdc42 or Rac1 may facilitate S6K1 activation by a membrane-bound kinase, such as PDK1 [54,55]. In light of the new PLD–PA–mTOR pathway, an alternative interpretation may involve Rho-mediated activation of PLD1, which in turn produces PA and activates mTOR signaling to S6K1. It should be noted, however, that RhoA does not activate S6K1 [53], although it activates PLD1 effectively. Whether Cdc42 and Rac1 activate S6K1 through PLD1 should be examined directly.

4.3. cPKC

cPKCs also directly interact with PLD1. Both kinase-dependent and kinase-independent mechanisms have been proposed for cPKC regulation of PLD1 [34,49,56]. 4β-Phorbol-12β-myristate-13γ-acetate (PMA) is known as a potent activator of S6K1 (see, for example, Ref. [52]), suggesting that a kinase belonging to the conventional or novel PKC class may be responsible for mediating the signaling, although other PMA effectors cannot be ruled out. The involvement of cPKC in the S6K1 pathway is further implicated by the inhibitory effect of a specific PKCα/β inhibitor on serum-stimulated S6K1 activation in HEK293 cells (unpublished observation). Thus, PLD1 and PA could potentially transduce signals from cPKC to S6K1 *in vivo*. Future experiments should probe a potential relationship of cPKC to PLD1 and S6K1. The array of PLD1 mutants generated by the Frohman laboratory [57,58] displaying selective response to upstream regulators should prove powerful in delineating the roles of PLD1, cPKC, Rho, and ARF in the PA–mTOR pathway.

4.4. Calcium ion

Another set of observations not explained previously is the participation of cellular Ca^{2+} in the activation of S6K1. Calcium ionophores and compounds that release intracellular calcium stores are found to activate S6K1 [36,52,59]. In liver epithelial cells, angiotensin II-induced S6K1 activation seems to require Ca^{2+} [59]. In addition, LPA stimulation of S6K1 in Swiss3T3 cells is blocked by a cell-permeable Ca^{2+} chelator [36]. These calcium effects may again be attributed to PLD1, since PLD1 activation has been reported to require calcium [34], although whether cPKC or other regulators mediate the calcium action remains to be determined. Alternatively, Ca^{2+} clustering of PA [60,61] may effectively raise the local concentration of PA, which in turn activates mTOR.

4.5. Other PA-generating enzymes

Although PLD appears to be the enzyme largely responsible for mitogen-stimulated PA in mTOR signal transduction in HEK293 cells, it is conceivable that some cell types may use alternative routes for PA synthesis. For instance, DGK phosphorylates DAG to yield PA. Nine isoforms of DGKs have been identified in mammals, which are classified into five subgroups based on their primary structures (reviewed in Ref. [62]). In HEK293 cells, the type I, Ca^{2+}-activated DGKs do not seem to contribute to mTOR

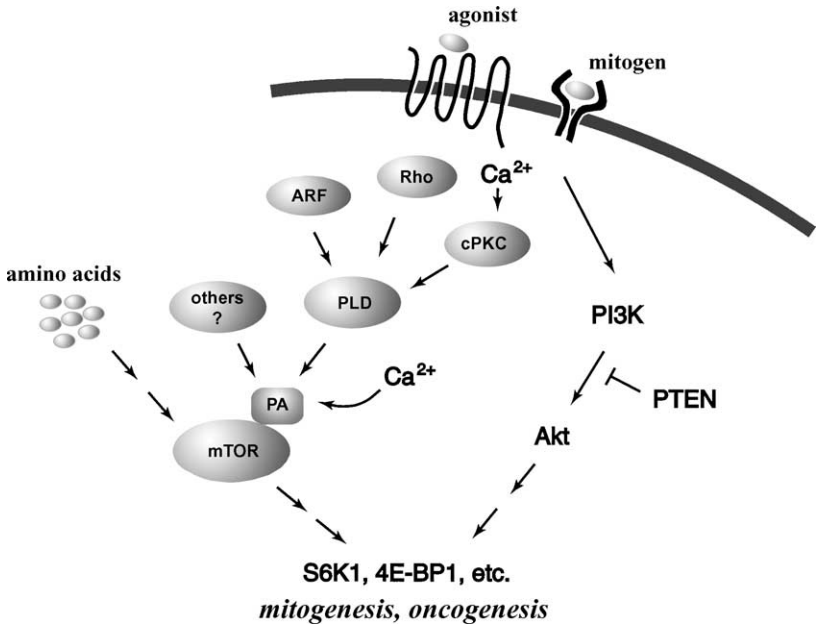

Fig. 2. Rapamycin-sensitive signaling circuitry: a working model.

signaling, as the specific inhibitor R59949 had no effect on S6K1 activation (unpublished observation). However, it cannot be ruled out that some of the nine mammalian DGKs with distinct cell and tissue distribution [62] may influence the mTOR pathway in certain cell types.

Another potentially relevant enzyme is LPAAT, which catalyzes the conversion of LPA to PA. The majority of the LPAAT activity in human cells can be attributed to two isoforms: LPAAT-α and LPAAT-β (reviewed in Ref. [63]). Although it is not clear how LPAAT activity may be regulated, increased LPAAT-β expression levels have been found to associate with many human tumors [63], suggesting that LPAAT-β may be involved in mitogenesis and oncogenesis. A potential link between LPAAT and mTOR signaling should be probed in future studies.

5. Concluding remarks

The identification of PA as a second messenger in mTOR regulation has uncovered a new pathway and revealed the role of mTOR to integrate both nutrient and mitogen signals to regulate downstream signaling. A mechanism for rapamycin inhibition *in vivo* is proposed, in which rapamycin inhibits the ability of mTOR to activate downstream effectors—but not its intrinsic catalytic activity—by blocking PA binding to the FRB domain. The implication of PLD in mTOR signaling brings to light several candidate regulators that potentially mediate a diverse range of mTOR regulation (Fig. 2). Awaiting future investigation is the potential involvement of cPKC, ARF, Rho, the calcium ion, and other regulators of PA production in mTOR signaling, in a cell type-specific and signal-specific context.

The PI3K pathway represents a major branch of oncogenic signaling [64]. Aberrant gain of function in this pathway due to amplified activation of PI3K or Akt, or loss of PTEN, is associated with numerous human cancers [2]. The mTOR pathway controls the downstream outcome of PI3K signaling by supplying critical input regarding amino acid availability (Fig. 2), and the inter-dependence of these two pathways is best demonstrated by the findings that rapamycin and its analogue CCI-779 display highly selective potency against tumors and transformed cell lines with hyperactive PI3K signaling [65–67]. The identification of new components and regulatory modes in the mTOR signaling circuitry promises to uncover novel drug targets and help develop new diagnostic and therapeutic strategies.

Acknowledgments

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