Regulation of mTOR by Phosphatidic Acid?

David A. Foster

Department of Biological Sciences, Hunter College of The City University of New York, New York, New York

Abstract

Interest in the regulation of the mammalian target of rapamycin (mTOR) has increased substantially in recent years largely because of an apparent link between mTOR and survival signals in human cancer cells. Much has been learned about the regulation of mTOR in response to survival signals generated by phosphatidylinositol 3-kinase (PI3K). However, another mechanism for regulating mTOR has been proposed involving the generation of phosphatidic acid (PA). PA is the metabolic product of phospholipase D (PLD), whose activity is elevated in a large number of human cancers, and, like PI3K, has been implicated in the survival of human cancer cells. Although the regulation of mTOR by the PI3K signaling pathway is well established, a role for PLD and PA in regulating mTOR has been controversial. In this review, the evidence implicating PLD and PA in the regulation of mTOR is summarized, and the implications of this novel and potentially important mechanism for regulating mTOR are discussed. [Cancer Res 2007;67(1):1–4]

Introduction

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates a variety of cellular activities that are sensitive to environmental stress (1). An emerging theme in tumorigenesis is that normal stress responses must be overcome in order for cancer cells to survive and proliferate, and it is becoming apparent that mTOR is a critical target of "survival signals" in cancer cells (2). For this reason, the signaling pathways that target mTOR have been aggressively studied and widely reviewed. mTOR is an indirect target of survival signals generated by phosphatidylinositol 3-kinase (PI3K). PI3K generates phosphatidylinositol 3,4,5-tris-phosphate (PIP3), which leads to the recruitment and activation of the Akt kinase. Akt then phosphorylates several substrates, one of which is the tuberous sclerosis complex (TSC1/2), a GTPase activating protein complex that suppresses Rheb, a GTPase that contributes directly to the activation of mTOR (Fig. 1*A*). Akt suppresses TSC1/2, leading to the activation of mTOR. On the other hand, TSC1/2 can also be targeted and activated by LKB1 tumor suppressor protein, activating the AMP-dependent kinase (2), which subsequently activates TSC1/2, resulting in the down-regulation of Rheb and subsequent inhibition of mTOR (Fig. 1*A*). Thus, there are signals that both activate and suppress mTOR through the TSC1/2 complex and Rheb. Although the regulation of mTOR through TSC1/2 and Rheb has been widely described, another mechanism contributing to the activation of mTOR has been proposed that has received less attention. Chen et al. reported a phosphatidic acid (PA) requirement for the ability of mTOR to phosphorylate S6 kinase (3). PA is most commonly generated by the hydrolysis of phosphatidylcholine by phospholipase D (PLD; ref. 4). Like PI3K, PLD has been implicated in survival signals in human cancer cells (5). mTOR was reported to bind PA in a manner that was competitive with rapamycin complexed with the FK506-binding protein (FKBP12; ref. 3). This finding lead Chen et al. to propose a model for the action of rapamycin whereby rapamycin/FKBP12 binds to mTOR, preventing interaction with PA (3, 6). Consistent with the proposal that the rapamycin/FKBP12 complex acts competitively with PA, elevated PLD activity in human breast cancer cells increased the concentration of rapamycin required to suppress mTOR (7). Although the model for the action of rapamycin as a competition with PA for mTOR is consistent with available data, a role for PA and PLD in the regulation of mTOR remains controversial. In this review, the evidence implicating PLD and its metabolic product PA in the regulation of mTOR is summarized, and the implications are discussed.

mTOR Regulation by PA

The PA connection. The first demonstration that PA was involved in the regulation of mTOR came from the lab of Jie Chen (3), who showed that exogenously provided PA stimulated the activation of the mTOR substrate S6 kinase and phosphorylation of another mTOR substrate eukaryotic initiation factor 4E binding protein-1 (4E-BP1) in HEK293 cells. The effect of PA was sensitive to rapamycin (3, 8) and was dependent on the presence of amino acids (3). The amino acid dependence indicated that the effect of PA was physiologic. Subsequently, Blenis' group similarly showed that PA stimulated S6 kinase activity in HEK293 cells (9). The ability of PA to stimulate S6 kinase was suppressed by coexpression of TSC1/2, also suggesting that the PA-induced S6 kinase activity was physiologic. PA was also shown to activate mTOR in macrophages in an Akt-dependent manner (10). In addition, Xu et al. (11) showed that mTOR could be activated by phosphonate analogues of PA as measured by increased S6 kinase activity. Thus, several laboratories have shown that mTOR is activated in response to exogenously supplied PA, and in two of these cases, there was another physiologic component other than PA that contributes to the regulation of mTOR, suggesting that the effects of PA were not artifacts.

A role for PLD-generated PA in the activation of mTOR. The major cellular mechanism for generating PA is through the hydrolysis of phosphatidylcholine by PLD. There are two mammalian PLD isoforms (PLD1 and PLD2) that can be distinguished by different mechanisms of regulation and subcellular distribution (4). PLD1 has a perinuclear localization and is regulated by Rho, Ral, and ARF family GTPases (4). PLD2 is largely restricted to lipid raft fractions on the plasma membrane, and its mode of regulation is not well understood (4). Both PLD1 and PLD2 have a stringent requirement for PIP2 (4). The production of PA by either PLD1 or

Requests for reprints: David A. Foster, Department of Biological Sciences, Hunter College of The City University of New York, 695 Park Avenue, New York, NY 10021. Phone: 212-772-4075; E-mail: foster@genectr.hunter.cuny.edu.

doi:10.1158/0008-5472.CAN-06-3016

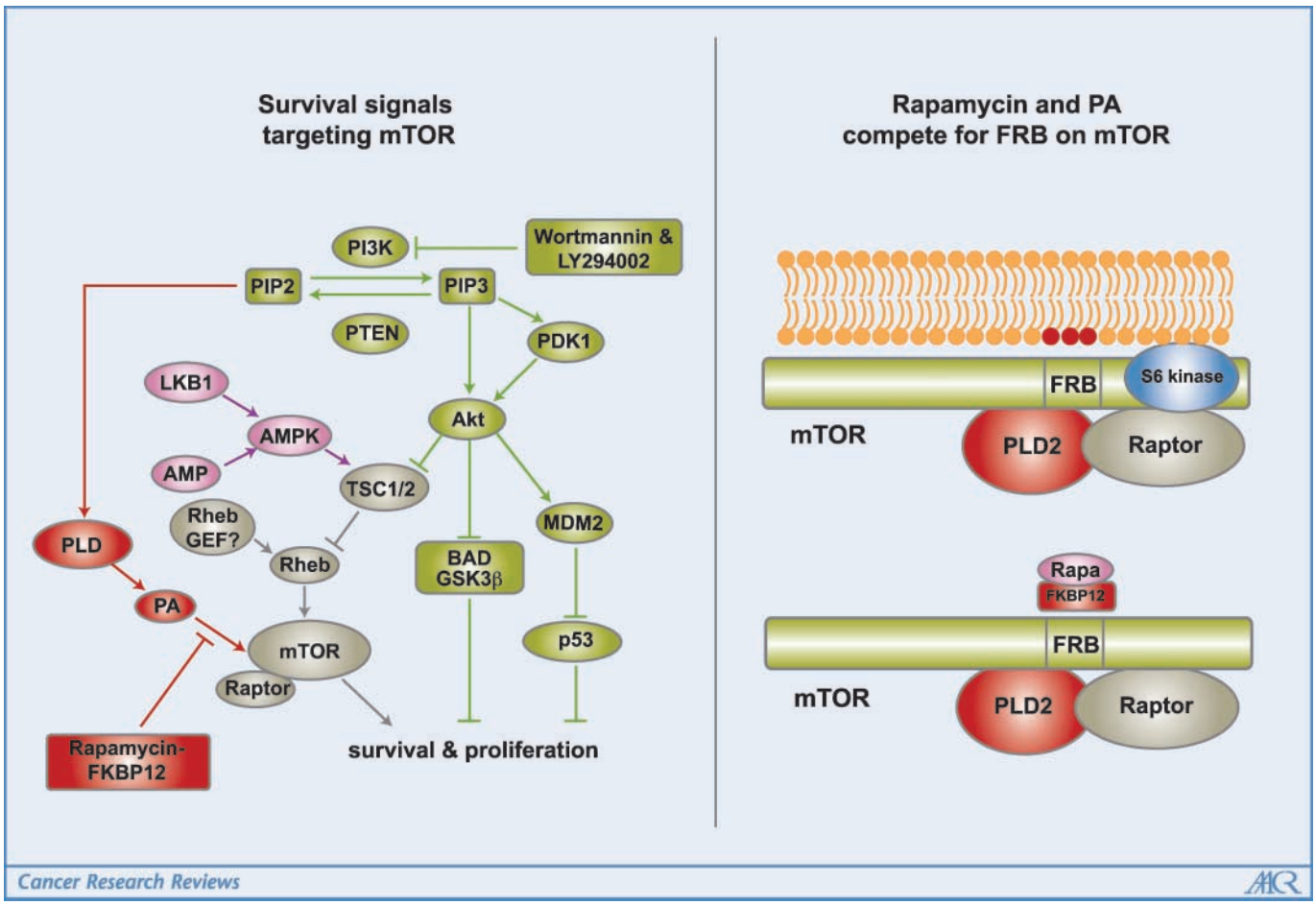

Figure 1. Regulation of mTOR. *Left panel,* mTOR is activated by the GTPase Rheb, which is negatively regulated by the TSC1/2 GTPase-activating complex of hammartin and tuberin. Two pathways feed into TSC1/2. The PI3K pathway activates Akt, which then phosphorylates several substrates that help to promote survival and proliferation, including MDM2, GSK3-β, BAD, and TSC1/2. The phosphorylation of TSC1/2 by Akt suppresses the GTPase-activating effects of TSC1/2 on Rheb and enhances mTOR activity. The LKB1 pathway begins with the tumor suppressor protein kinase LKB1, which contributes to the activation of the AMP kinase (*AMPK*), which then phosphorylates TSC1/2, leading to the activation of TSC1/2 GTPase-activating activity and the suppression of mTOR. The PLD pathway is proposed to contribute to the activation of mTOR by generating PA from phosphatidylcholine, which then binds the FRB domain in a manner competitive with rapamycin/FKBP12. *Top, right panel* mTOR in complex with raptor and PLD2 associates with PLD2-generated PA (*red circles*) through its FRB domain. In the presence of rapamycin (*Rapa*)/FKBP12 complex (*bottom, right panel*), the FRB domain of mTOR is unable to bind PA, and mTOR is unable to access and phosphorylate appropriate substrates, such as S6 kinase. Excess levels of PA on the membrane would provide more sites for binding mTOR and compete with rapamycin/FKBP12 complexes.

PLD2 can be suppressed by primary, but not tertiary, alcohols in what is known as the transphosphatidylation reaction whereby an inert phosphatidyl-alcohol is generated rather than PA. This reaction has been widely used to indicate a requirement for PLD, and several studies have indicated that the activation of mTOR was sensitive to primary alcohols. Serum-induced increases in S6 kinase activity and 4E-BP1 phosphorylation were blocked by 1-butanol in HEK293 cells (3). Phenylephrine (α-adrenergic receptor agonist)–induced mTOR was also sensitive to treatment with 1-butanol (12). In skeletal muscle, PA stimulated S6 kinase phosphorylation, and 1-butanol suppressed S6 kinase phosphorylation (13). A very recent report showed a nutrient-dependent multimerization of mTOR that was also suppressed by 1-butanol (14). Thus, suppression of PLD-generated PA with primary alcohols has been reported to suppress the effects of mTOR in several systems, cell types, and cellular phenotypes.

In addition to experiments that used primary alcohols to suppress PLD-induced increases in PA, several studies have investigated the dependence mTOR activation on PLD1 and PLD2 expression. Exogenously expressed PLD2 was shown to increase S6 kinase phosphorylation in MCF7 cells (15), and elevated expression of PLD1 was reported to increase S6 kinase phosphorylation in rat fibroblasts (16). Lysophosphatidic acid (LPA)–induced mTOR was shown to be dependent on PLD1 and Rho (17). The activation of mTOR by Cdc42 was also dependent on PLD1 (18). Suppression of PLD1 expression blocked S6 kinase phosphorylation in B16 melanoma cells, and suppression of either PLD1 or mTOR led to melanogenesis in these cells (19). Both PLD1 and PLD2 were able to suppress protein phosphatase 2A (PP2A) in a rapamycin-dependent manner (20). PLD2 also suppressed the association between PP2A and the mTOR substrates S6 kinase and 4E-BP1 (20). Very recently, it was reported that PLD2 forms a functional complex with mTOR and its binding partner Raptor (known as mTORC1) through a TOS (TOR signaling) motif in PLD2 (21). The interaction between mTORC1 and PLD2 was essential for mitogen stimulation of mTOR (21). Thus, in addition to the ability of PA to activate mTOR, there are several studies that have shown a PLD requirement for the activation of mTOR. Although it is not clear

how both PLD1 and PLD2 contribute to the activation of mTOR, it has been proposed that elevated PLD1 leads to the activation of PLD2 by increasing levels of PIP2 required for the activity of PLD2 (5). This could explain the apparent involvement of both PLD1 and PLD2 in the activation of mTORC1.

Other mechanisms for elevating PA. In addition to PLD, it has been suggested that PA generated by other metabolic pathways could contribute to the activation of mTOR. It has also been reported that suppression of LPA acyltransferase (LPAAT), which generates PA by acylating LPA, suppressed mTOR activation (22). The suppression of LPAAT activity disrupted survival and proliferative signals in several cancer cell lines (22). Similarly, it was reported that mTOR is activated in response to diacylglycerol kinase (23), which generates PA by phosphorylating diacylglycerol. These studies further establish that elevated PA levels in cells leads to the activation of mTOR and suggest the possibility that there are alternative mechanisms for generating the PA required for activating mTOR. There have been concerns that studies with exogenously provided PA activates mTOR artifactually because it is difficult for PA to get across the membrane to appropriate sites where mTOR could be activated. Although evidence that firmly establishes a role for LPAAT and diacylglycerol kinase in the physiologic activation of mTOR is still missing, the ability of elevated LPAAT and diacylglycerol kinase activity to stimulate mTOR provides evidence that PA generated physiologically inside of cells can lead to the activation of mTOR. Additionally, because PA can be metabolically converted to either LPA or diacylglycerol (4), these studies also indicate that it is PA, and not metabolites of PA, that is acting on mTOR.

Dependence of PLD-induced mTOR activation on PI3K activity? Elevated PLD activity, under most circumstances, does not lead to increased phosphorylation of Akt. Increased expression of PLD1 led to increased S6 kinase phosphorylation but did not increase Akt phosphorylation in rat fibroblasts (16). However, PLD1-induced increases in S6 kinase phosphorylation were blocked by the PI3K inhibitor LY294002 (16). Similarly, LY294002 suppressed S6 kinase phosphorylation in MDA-MB-231 cells, where there is elevated expression of PLD1 and elevated PLD activity (15). However, Tee et al. (9) were able to activate mTOR with exogenously supplied PA in the presence of the PI3K inhibitor wortmannin. These data indicate that whereas PLD and PA can stimulate mTOR, basal levels of PI3K activity and the suppression of TSC1/2 may be required.

Are the effects of rapamycin on mTOR due to competition with PA? Rapamycin interacts with FKBP12, which then interacts mTOR in a region known as the FKBP12-rapamycin-binding (FRB) domain. Chen et al. proposed a model for the mechanism of rapamycin suppression of mTOR based their observation that the FRB domain of mTOR bound PA in a manner that was competitive with rapamycin/FKBP12 (3, 6). It was found that the FRB domain of mTOR bound to phosphatidylcholine-based vesicles containing as little as 10% PA but not to vesicles containing other phospholipids, including phosphatidylserine, phosphatidylethanolamine, PI, PIP, PIP2, and PIP3 (3). The binding of the FRB domain to PA was blocked by FKBP12/rapamycin, indicating a competition between PA and FKBP12/rapamycin for the FRB domain of mTOR. Moreover, an FRB domain from a rapamycin-resistant mTOR mutant bound PA in a rapamycin-resistant manner (3). Mutation of Arg^{2109} seemed to be critical for the interaction between the FRB and PA (3). Moreover, expression of this mutant in HEK293 cells suppressed serum-induced S6 kinase phosphorylation (3). Based on these data, it was proposed that the basis for rapamycin action is due to preventing interaction with PA (6). Consistent with this hypothesis, elevated PLD activity in MDA-MB-231 and MCF7 human breast cancer cells increased the IC_{50} for rapamycin. The IC_{50} for the suppression of cell proliferation by rapamycin in MDA-MB-231 cells, which have high levels of PLD activity, was 10 μmol/L, whereas in MCF7 cells, which have low levels of PLD activity, it was 20 nmol/L (7). Interestingly, the IC_{50} for S6 kinase phosphorylation in MDA-MB-231 cells was 20 nmol/L and 1 nmol/L in MCF7 cells (7). Thus, although lower concentrations of rapamycin were required to suppress S6 kinase phosphorylation than was required to suppress proliferation, higher level of PLD still increased the IC_{50} for the effect of rapamycin. Elevated expression of PLD2 increased IC_{50} for rapamycin in MCF7 cells, and expression of a dominant-negative PLD2 decreased the IC_{50} for rapamycin in MDA-MB-231 cells (7). In skeletal muscle, mechanical stimulation led to elevated PLD activity and also increased the IC_{50} for rapamycin (13). These data are all consistent with the model proposed by Chen and Fang (6), whereby the effects of rapamycin are due to interfering with an interaction between the FRB of mTOR and PLD generated PA (shown schematically in Fig. 1*B*). It has been suggested that the mechanism of rapamycin action is simply to alter the structure of mTOR (1), and whereas this is certainly also possible, the data indicating a competition between PA and rapamycin/FKBP12 for mTOR represents a compelling alternative that is supported by both *in vitro* and *in vivo* studies. It is also possible that rapamycin/FKBP12 changes the conformation of mTOR and suppresses interaction with PA. Another possibility is that the PA generated by PLD could reduce the pH locally such that mTOR gets protonated in such a way the mTOR kinase activity is increased or the association with substrates is altered.

Summary, Significance, and Directions

Elevated PLD activity has been implicated in a large number of human cancers, including breast, colon, gastric, and kidney (5). PLD activity has been reported to suppress apoptosis in human cancer cells subjected to the stress of serum withdrawal (5). In fact, PLD activity is elevated in several cancer cells in response to the stress of serum withdrawal (24). mTOR has been implicated as a key regulator of stress responses by shutting down under conditions of poor nutrition or hypoxia. For a cancer cell to survive and proliferate, it is becoming apparent that it must overcome normal cellular responses to stress to continue proliferation in an emerging tumor with poor vascularization. Elevated PLD activity observed in response to serum withdrawal in cancer cells may be a mechanism to activate mTOR that is genetically selected for to overcome the shutdown of mTOR in response to stress.

Collectively, the data linking PLD-generated PA production with mTOR activation represent a novel and potentially important mechanism for activating this critical regulator of responses to environmental stress and survival signals in cancer. The significant percentage of cancers with elevated PLD activity strongly suggest that this pathway for activating mTOR be carefully considered especially because targeting mTOR with rapamycin in cancer would be strongly influenced by the level of PLD activity in the cancer cells. Elevated PLD activity confers resistance to rapamycin (7, 13), which could have important unintended clinical consequences in that rapamycin treatment could actually select for cancer cells with

elevated PLD activity. The elevated PLD activity would not only generate rapamycin resistance, it could also make cells more malignant because elevated PLD activity also stimulates the metastatic phenotypes of increased cell migration and invasion (24). Thus, the potential for PLD to affect the targeting mTOR in multiple ways in anticancer therapies argues strongly that serious attention be given to the role of PLD and PA in the regulation of mTOR, especially with regard to targeting mTOR in anticancer therapies.

Future studies to establish a role of PLD for in the regulation mTOR will include studies on the subcellular distribution of PLD isoforms and mTOR and on the molecular mechanism through which PA affects mTOR. It will be critical to evaluate the subcellular localization of the participants in the regulation of mTOR. PLD2, which has recently been shown to associate with mTOR-Raptor complex (21), localizes primarily with lipid raft fractions on the plasma membrane, whereas PLD1 localizes primarily to perinuclear membranes (4, 5). We have found that in MDA-MB-231 breast cancer cells, mTOR has a perinuclear distribution similar to that of PLD1.[1] However, PLD1 has also been found on the plasma membrane, and PLD2 has been found in the nucleus (5, 6). Thus, clearly there is a need to evaluate the subcellular distribution of PLD isoforms, mTOR, and other regulators of mTOR in various cell lines where mTOR is active. Further studies are also needed to establish the precise mechanism of PA action on mTOR to determine exactly how PA affects mTOR signaling. It will also be of interest to establish whether PA has any effect on the rapamycin-resistant mTORC2 complex of mTOR and Rictor (25). Recent work from Sabatini et al. has shown that the formation of a complex between Raptor and mTOR (known as mTORC1) is disrupted by rapamycin (26). If rapamycin is acting to suppress interaction between mTOR and PA as proposed (6), then the role of PA may be to facilitate the interaction between mTOR and Raptor. Raptor has been implicated in regulating substrate specificity of mTOR (1). If PA does affect the mTOR-Raptor interaction, then PA could affect substrate recognition and not the kinase activity of mTOR. This would be consistent with unpublished data from Chen et al. (3, 6) indicating that PA does not affect the kinase activity of mTOR *in vitro*. More mechanistic details on the interaction of PA with mTOR could provide insights into the generation of rapamycin derivatives that are not as sensitive to PA levels. This will be especially important for the development of strategies for targeting the large percentage of human cancers where mTOR is providing survival signals, especially those being fueled by PLD-generated PA.

[1] Our unpublished results.

Acknowledgments

Received 8/14/2006; revised 9/24/2006; accepted 10/26/2006.

Grant support: NIH/National Cancer Institute grant CA46677 (D.A. Foster), NIH Support of Continuous Research Excellence grant GM60654 (D.A. Foster), and NIH/Research Centers in Minority Institutions grant RR-03037 (infrastructure and instrumentation in the Biological Sciences Department at Hunter College).

I thank John Blenis and David Sabatini for challenging discussions and constructive comments on the article and Greg Hoffman for critical comments on the article.

References

1. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol 2005;17:596–603.
2. Tee AR, Blenis J. mTOR, translational control and human disease. Semin Cell Dev Biol 2005;16:29–37.
3. Fang Y, Vilella-Bach M, Bachmann R, et al. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 2001;294:1942–5.
4. Exton JH. Phospholipase D-structure, regulation and function. Rev Physiol Biochem Pharmacol 2002;144:1–94.
5. Foster DA. Phospholipase D survival signals as a therapeutic target in cancer. Curr Signal Transduction Ther 2006;1:295–303.
6. Chen J, Fang Y. A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. Biochem Pharmacol 2002;64:1071–7.
7. Chen Y, Zheng Y, Foster DA. Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene 2003;22:3937–42.
8. Park IH, Bachmann R, Shirazi H, Chen J. Regulation of ribosomal S6 kinase 2 by mammalian target of rapamycin. J Biol Chem 2002;277:31423–9.
9. Tee AR, Anjum R, Blenis J. Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin. J Biol Chem 2003;278:37288–96.
10. Lim HK, Choi YA, Park W, et al. Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. J Biol Chem 2003;278:45117–27.
11. Xu Y, Fang Y, Chen J, Prestwich GD. Activation of mTOR signaling by novel fluoromethylene phosphonate analogues of phosphatidic acid. Bioorg Med Chem Lett 2004;14:1461–4.
12. Ballou LM, Jiang YP, Du G, Frohman MA, Lin RZ. Ca(2+)- and phospholipase D-dependent and -independent pathways activate mTOR signaling. FEBS Lett 2003;550:51–6.
13. Hornberger TA, Chu WK, Mak YW, Hsiung JW, Huang SA, Chien S. The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc Natl Acad Sci U S A 2006;103:4741–6.
14. Takahara T, Hara K, Yonezawa K, Sorimachi H, Maeda T. Nutrient-dependent multimerization of the mammalian target of rapamycin through the N-terminal HEAT repeat region. J Biol Chem 2006;281:28605–14.
15. Chen Y, Rodrik V, Foster DA. Alternative phospholipase D/mTOR survival signal in human breast cancer cells. Oncogene 2005;24:672–9.
16. Hui L, Abbas T, Pielak R, Joseph T, Bargonetti J, Foster DA. Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53. Mol Cell Biol 2004;24:5677–88.
17. Kam Y, Exton JH. Role of phospholipase D1 in the regulation of mTOR activity by lysophosphatidic acid. FASEB J 2004;18:311–9.
18. Fang Y, Park IH, Wu AL, et al. PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr Biol 2003;13:2037–44.
19. Ohguchi K, Banno Y, Nakagawa Y, Akao Y, Nozawa Y. Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells. J Cell Physiol 2005;205:444–51.
20. Hui L, Rodrik V, Pielak RM, Zheng Y, Foster DA. mTOR-dependent suppression of protein phosphatase 2A is critical for phospholipase D survival signals in human breast cancer cells. J Biol Chem 2005;280:35829–35.
21. Ha SH, Kim DH, Kim IS, et al. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. Cell Signal 2006;18:2283–91. Epub 2006 Jun 3.
22. Coon M, Ball A, Pound J, et al. Inhibition of lysophosphatidic acid acyltransferase-β disrupts proliferative and survival signals in normal cells and induces apoptosis of tumor cells. Mol Cancer Ther 2003;2:1067–78.
23. Avila-Flores A, Santos T, Rincon E, Merida I. Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. J Biol Chem 2005;280:10091–9.
24. Zheng Y, Rodrik V, Toschi A, et al. Phospholipase D couples survival and migration signals in response to stress in human breast cancer cells. J Biol Chem 2006;281:15862–8.
25. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 2006;6:729–34.
26. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 2004;14:1296–302.

Regulation of mTOR by Phosphatidic Acid?

David A. Foster

Cancer Res 2007;67:1-4.