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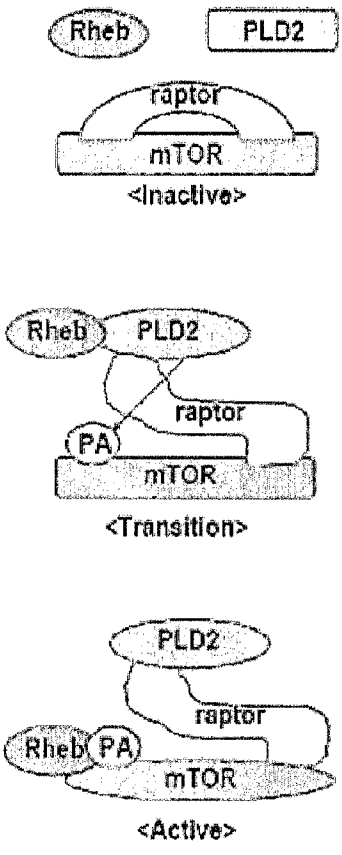
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(54) Title: METHOD OF REGULATING MAMMALIAN TARGET-OF-RAPAMYCIN ACTIVITY BY INTERACTION BETWEEN PHOSPHOLIPASE D AND RHEB



(57) Abstract: The present invention relates to method of regulating mammalian target-of- rapamycin (mTOR) based on a novel finding of a regulating mechanism of mTOR by a phospholipase D (PLD), and Ras homolog enriched in brain (Rheb). Further, the present invention also relates to a method of screening inhibitors of mTOR, and a method and a composition for treating mTOR-related metabolic diseases by inhibitin mTOR.

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TITLE OF THE INVENTION

METHOD OF REGULATING MAMMALIAN TARGET-OF-RAPAMYCIN ACTIVITY BY INTERACTION BETWEEN PHOSPHOLIPASE D AND RHEB

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to and the benefit of Provisional Application No. 60/821,542 filed on August 4, 2006, which is hereby incorporated by reference for all purposes as if fully set forth herein.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to method of regulating mammalian target-of-rapamycin (mTOR) based on a novel finding of a mechanism of regulating mTOR by a phospholipase D (PLD), and a Ras homolog enriched in brain (Rheb). Further, the present invention also relates to a method of screening inhibitors of mTOR, and a method and a composition for treating mTOR-related metabolic diseases by inhibiting mTOR.

(b) Description of the Related Art

mTOR is a serine/threonine protein kinase and a member of a novel superfamily of signaling proteins termed PI 3-kinase related kinases (PIKKs), based on sequence similarity of their catalytic domains. The mTOR pathway is an emerging target for the treatment of cancer, diabetes, obesity, hamartoma syndromes, tissue/organ hypertrophy, etc. Recent studies have demonstrated its role as a mediator of lifespan control in *C.elegans* and Drosophila. Despite the significance of this pathway in such diverse biological processes, the mechanism of its regulation by nutrients remains unknown.

In addition, mTOR requires the lipid second messenger phosphatidic acid (PA) for its activation. PA is an enzymatic product of PLD. PLD, which hydrolyzes phosphatidylcholine (PC) to generate PA, constitutes another branch of the mTOR upstream regulators through which mitogenic signals impinge on the

mTOR pathway. Mammalian PLD isozymes identified to date, PLD1 and PLD2, sense a variety of signals, such as neurotransmitters, hormones and growth factors, to regulate multiple physiological events such as proliferation, secretion, respiratory burst and actin cytoskeletal reorganization, and the like.

PA binds to mTOR and activates its activity to phosphorylate S6K1 and 4EBP1, two known downstream effectors of mTOR19. However, the mechanism by which PA activates mTOR in cells remains unknown. Recently, the inventors found that PLD2 specifically forms a functional complex with the mTOR/raptor complex to transducer mitogenic signals, and suggested that the localized generation of PA is essential for PLD2 activation of mTOR kinase activity as provided by the interaction with raptor through TOS motif in PLD2 (Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291, which is hereby incorporated by reference). The above identification of PLD2 as a functional and physical mediator for the mTOR/raptor complex led the inventors to test the interrelations between PLD2 and Rheb, to complete the present invention.

SUMMARY OF THE INVENTION

An aspect of the present invention is to reveal a mechanism of regulating mTOR activity by PLD and Rheb. More specifically, the mTOR activity may be activated by the steps of

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to activate the mTOR kinase activity.

Based on the above, another aspect of the present invention is to provide a method of regulating mTOR activity by regulating one or more steps among the following steps:

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to activate the mTOR kinase activity.

Another aspect of the present invention is to provide a method of screening inhibitors of mTOR activity. The method of screening inhibitors of mTOR according to the present invention may comprising the steps of:

contacting a candidate compound to a sample cell;

examining the interaction between PLD2 and Rheb by which Rheb is capable of binding to mTOR through PLD2; and

determining the compound as an inhibitor of mTOR activity when the level of the interaction between PLD2 and Rheb decreases compared with that in other sample cells without contacting with the compound.

Another aspect of the present invention is to provide the amino acid sequence from position 476 to position 612 of full-length PLD2 as a target for screening of an agent of treating mTOR-related metabolic diseases may include cancer, diabetes, obesity, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

Still other aspect of the present invention is to provide methods and compositions for treating mTOR-related metabolic diseases by inhibiting mTOR activity.

The method of treating mTOR-related metabolic diseases may be conducted by inhibiting one or more steps among the steps of:

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to activate the mTOR kinase activity.

The composition may contain an effective amount of an inhibitor of mTOR as an active ingredient.

The mTOR-related metabolic diseases may include cancer, diabetes, obesity,

hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the immunoblotting results from knockdown PLD1 and/or PLD2.

Figure 2 shows the level of posphatidyl-butanol formation (PBt formation) from knockdown PLD1, PLD2, and/or Rheb.

Figure 3 shows the results of western blot analysis from HA-Rheb^{wt} transfected with PLD2 or PLD1.

Figure 4 shows the results of GST-pull down analysis with PLD2-expressing cell lysates.

Figure 5 shows a schematic view of the PLD2 fragments (upper panel) and the result of GST-pull down analysis using the PLD2 fragments (lower panel).

Figure 6 shows the results of western blot analysis using PLD2 fragments, 2F3 and 2F2.

Figure 7 shows the results of co-IP (immunoprecipitation) against anti-mTOR antibody.

Figure 8 shows the levels of bound raptor and PLD2 detected by Western blotting using various PLD mutants.

Figure 9 shows the IP results against anti-HA antibody in mTOR/raptor complex.

Figure 10 shows the co-IP analysis results against anti-HA antibody in HA-Rheb^{Q64L} transfected with GFP-2F3 or GFP-2F2.

Figure 11 shows the results of mTOR kinase assays using anti-myc immunoprecipitates.

Figure 12 shows the IP analysis results obtained in the transfected HA-RhebQ64L treated with 1-butanol, t-butanol, phosphatidic acid, and rapamycin, respectively.

Figure 13 shows IP analysis results obtained from the binding of GST-Rheb to mTOR complex by PA in the presence of GDPβS or GTPγS.

Figure 14 shows the results of *in vitro* mTOR kinase assay for myc-mTOR

transfectant.

Figure 15 shows the results of *in vitro* mTOR kinase assay in COS7 cells transfected with HA-mTOR^{wt} and myc-Rheb^{wt} or myc-Rheb^{D60I}.

Figure 16 shows the level of PBt formation in presence or absence of leucine.

Figure 17 shows IP analysis results using anti-mTOR antibody in COS7 and HEK293 cells in presence or absence of leucine.

Figure 18 shows the western blot analysis results in HA-raptor^{wt}/PLD2^{wt} transfectant after leucine treatment.

Figure 19 shows the western blot analysis results in HA-Rheb^{wt}/PLD2^{wt} transfectant after leucine treatment.

Figure 20 shows the western blot analysis results in PLD2 siRNA transfectant after leucine treatment.

Figure 21 shows the western blot analysis results in HA-Rheb^{Q64L} transfected with either GFP-2F3 or GFP-2F2.

Figure 22 shows the changes in cell size after transfecting various PLD2 mutants.

Figure 23 shows a working model for the cooperation of PLD2 and Rheb in nutrient-induced mTOR activation, explaining the role of PLD2 in the Rheb activation of mTOR signaling.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A more complete appreciation of the invention, and many of the attendant advantages thereof, will be readily apparent as the same becomes better understood by reference to the following detailed description.

The inventors speculate that; the mTOR/raptor/PLD2/Rheb complex is stabilized in leucine-rich conditions, PLD2 is activated by Rheb binding, generated PA increases Rheb binding to mTOR, and that finally mTOR is activated. The speculations were proved by the experimentations as below, thereby completing the present invention.

The present inventors have been studied the regulatory mechanisms of mTOR signaling, and found the participation of the mTOR complex (mTOR/raptor) containing raptor and GβL in response to upstream signals for the appropriate control

of cell growth. These upstream signals derived from insulin and/or nutrients are likely to be mediated by the tuberous sclerosis complex 1/2 and Rheb. In addition, mTOR requires the lipid second messenger phosphatidic acid (PA) for its activation. The present invention identifies the regulators of mTOR activity, and the physical/functional connections thererbetween, providing further insight into mTOR-related metabolic diseases such as cancer, diabetes obesity, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

Definition

The term 'mTOR' refers to a mammalian target-of-rapamycin. In the present invention, mTOR may be originated from any mammalians including human and its amino acid sequences according to the source species are well known in the relevant art. In the present invention, the mTOR may originated from any mammalians, for example, *Homo sapiens* (NP 004949), *Drosophila melanogaster* (NP524891), *Caenorhabditis elegans* (Q95Q95), etc. In an embodiment of the present invention, human mTOR having the amino acid sequence of SEQ ID NO: 1 may be used.

The term 'PLD' refers to a phospholipase D, and mammalian PLD isozymes include to classes, PLD1 and PLD2. In the present invention, PLD may be originated from any mammalians including human, and its amino acid sequences according to the source species are well known in the relevant art. In an embodiment of the present invention, PLD1 (NM 030992, originated from *Rattus norvegicus*) having the amino acid sequence of SEQ ID NO: 2, and PLD2 (NM 002663, originated from *Homo sapiens*) having the amino acid sequence of SEQ ID NO: 3 may be used.

The term 'PA' refers to a phosphatidic acid, which is an enzymatic product of PLD. mTOR requires the lipid second messenger phosphatidic acid (PA) for its activation.

The term 'Rheb' refers to a Ras homolog enriched in brain. In the present invention, the raptor may be originated from any mammalians including human, mouse, etc., and its amino acid sequences according to the source species are well known in the relevant art. In an embodiment of the present invention, the raptor

may be originated from human, and have the amino acid sequence of SEQ ID NO: 4 (Accession No. NP 065812).

The term 'raptor' refers to a regulatory-associated protein of mTOR. In the present invention, the raptor may be originated from any mammalians including human and its amino acid sequences according to the source species are well known in the relevant art. In an embodiment of the present invention, the raptor may be originated from human, and have the amino acid sequence of SEQ ID NO: 5 (Accession No. Q8N122).

An aspect of the present invention is to reveal a mechanism of regulating mTOR activity by PLD and Rheb. More specifically, the mTOR activity may be activated by the steps of

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to activate the mTOR kinase activity.

The mechanism is schematically shown in Fig. 23. Therefore, the mTOR activity may be regulated or inhibiting by regulating or inhibiting one or more steps among above steps 1) to 3).

The binding of Rheb to mTOR may be enhanced by PA in the presence of GTPγS, and thus inhibited by removing PA or GTPγS. Further, the interaction between PLD2 and Rheb may be regulated by nutrient levels, preferably amino acid level, more preferably leucine level, such that this interaction is stabilized under high nutrient conditions and weakened under low nutrient conditions. Therefore, the interaction between PLD2 and Rheb may be inhibited by decreasing the level of nutrients, preferably amino acids, more preferably leucine. As the similar manner, nutrient levels may also regulate the interaction between PLD2 and raptor that mediates the binding of PLD2 to mTOR, allowing a nutrient-dependent mTOR complex composed of mTOR, raptor, PLD2, and Rheb, as shown in Fig. 19.

Based on the above, another aspect of the present invention is to provide a method of regulating mTOR activity by regulating one or more steps among the

following steps:

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to activate the mTOR kinase activity.

The binding step of Rheb to mTOR may be regulated or inhibited by regulating or inhibiting the interaction between PLD2 and Rheb. The regulation or inhibition of the interaction between PLD2 and Rheb may be conducted by modifying a Rheb binding site of PLD2 and/or a PLD2 binding site of Rheb. In a preferable embodiment of the present invention, the Rheb binding site of PLD2 may comprise the amino acid residues from position 476 to position 612 of full-length PLD2. Therefore, the inhibition of the interaction between PLD2 and Rheb may be conducted by modifying the Rheb binding site of PLD2, and the modification may be deletion of one or more amino acids selected from the amino acid residues from position 476 to position 612 of full-length PLD2, or substitution one or more amino acids from the amino acid residues with other amino acid(s). Alternatively, the modification of Rheb binding site of PLD2 may be conducted by change of pH or temperature, and the like.

The method of inhibiting mTOR according to the present invention results in inhibiting the mTOR' phosphorylation activity on one or more mTOR effectors selected from the group consisting of ribosomal protein S6 kinase 1 (S6K1; e.g., NP 003152, NP 082535, etc.), and 4E-binding protein-1 (4EBP1; e.g., NP 004086).

Another aspect of the present invention is to provide a method of screening inhibitors of mTOR activity. The method of screening inhibitors of mTOR according to the present invention may comprising the steps of:

contacting a candidate compound to a sample cell;

examining the interaction between PLD2 and Rheb by which Rheb is capable of binding to mTOR through PLD2; and

determining the compound as an inhibitor of mTOR activity when the level of the interaction between PLD2 and Rheb decreases compared with that in other sample cells without contacting with the compound.

The sample cell may be any cell capable of expressing PLD2, more preferably PLD2. For example, the sample cell may be selected from the group consisting of a human embryonic kidney (HEK293), a human epithelial ovarian cancer cell (OVCAR-3), COS7 cell, a human cervical cancer (HeLa) cell, a human colon cancer cell (PC-3), a human breast cancer cell (MB231), a human hepatoma (HepG2), a human breast cancer cell (MCF-7), a human T cell leukemia (Jurkat), and the like. The inhibitor of mTOR may be useful in treating mTOR-related metabolic diseases, such as cancer, diabetes, obesity, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc. Therefore, the method of the present invention may also used in screening agents of mTOR-related metabolic disease selected from the group consisting of cancer, diabetes, obesity, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

The interaction between PLD2 and Rheb may be examined by any conventional method, for example by immunoprecipitation, but not limited thereto.

Another aspect of the present invention is to provide the amino acid sequence from position 476 to position 612 of full-length PLD2 as a target polypeptide for screening of an agent of treating mTOR-related metabolic diseases may include cancer, diabetes, obesity, hamartoma syndromes including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

Still other aspect of the present invention is to provide methods and compositions for treating mTOR-related metabolic diseases by inhibiting mTOR activity.

The method of treating mTOR-related metabolic diseases may be conducted by inhibiting one or more steps among the steps of:

1) binding of Rheb to mTOR through PLD2 by interaction between PLD2 and Rheb;

2) movement of PA produced from PLD2 near mTOR, to increase the level of PA near mTOR; and

3) binding of Rheb to mTOR due to the increase of PA near mTOR, to

activate the mTOR kinase activity.

More specifically, the method of treating mTOR-related metabolic diseases may be conducted by inhibiting the interaction between PLD2 and Rheb, thereby inhibiting the binding of Rheb to mTOR through PLD2. The treating method may comprise the step of inactivating the Rheb binding domain of PLD2, thereby inhibiting binding of Rheb to mTOR through PLD2. The inactivation of the Rheb binding domain of PLD2 is as aforementioned. Alternatively, the treating method comprises the step of administering an effective amount of an inhibitor of mTOR activity as an active ingredient, wherein the inhibitor of mTOR may be screened by the screening method according to the present invention.

The composition may contain an effective amount of an inhibitor of mTOR as an active ingredient. The inhibitor of mTOR activity may be any material having the activity to inhibit one or more steps among the above three steps. In an embodiment of the present invention, the inhibitor of mTOR activity may be any material having the activity to prevent PLD2 from binding to Rheb, thereby inhibiting the binding of Rheb to mTOR through PLD2, as aforementioned. The inhibitor of mTOR activity may be any material capable of inactivating the Rheb binding domain of PLD2 by various means as aforementioned. Alternatively, the inhibitor of mTOR activity may be a compound screened by the screening method according to the present invention.

The mTOR-related metabolic diseases may include cancer, diabetes, obesity, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc.

In the present invention, it is found that PLD2 interacts directly with Rheb and this is important for PLD2 activation. Importantly, this functional connection increases Rheb binding onto the mTOR/raptor complex and the generated PA increases Rheb activation of mTOR kinase activity. Therefore, it may be suggested that PLD2 and Rheb cooperate to regulate a nutrient-induced mTOR signaling.

Based on the above findings, a hypothetic model may be provided in which raptor binding to PLD2, and allowing the accumulation of PA near mTOR through Rheb-induced PLD2 activation, is a prerequisite for mTOR activation by Rheb

through the PA-dependent binding of Rheb to mTOR. The model is schematically shown in Fig. 23. Although it is not clear whether the interaction between PLD2 and Rheb is a first-going event for PLD2 binding with raptor, it is also possible that an unidentified signal to mTOR/raptor directly causes PLD2/Rheb to bind raptor since reducing PA generation or PLD2 expression did not modulate the raptor/mTOR interaction.

PLD1 as well as PLD2 activates the mTOR pathway. However, mTOR is likely to interact with PLD2 only, which implies an alternative pathway for the PLD1-dependent activation of the mTOR pathway, possibly through Cdc42/S6K1 signaling. The findings of the present invention also show that the silencing effect of PLD1 on mTOR signaling is completely rescued by PA treatment, but not in the case of silencing PLD2, suggesting an obvious difference between PLD1 and PLD2. The role of PLD1 in mTOR signaling might be mediated by solely PA generation. This also implies that the other PA target upstream of mTOR may be required to mediate this effect. It is possible that PLD2 is under the control of PLD1 since PLD1 signals PLD2 through phosphoinositide 4-phosphate 5 kinase.

PA has been recognized as lipid second messenger generated by mitogenic signals. To identify the downstream effectors, various PA-binding proteins have been found. These include Raf-1, sphingosine kinase 1, phosphatase PP2A, and phosphodiesterases 4A1 as well as mTOR. However, the action mechanism of PA on these effects remains to be addressed. In the present invention, it is of interest to examine the role of PLD2 in the regulation of the other PA binding proteins.

Knowledge about the molecular mechanism by which the mTOR pathway is regulated by cellular nutritional states and how impairment of the pathway leads to metabolic diseases, such as cancer, obesity, diabetes, hamartoma syndrome including tuberous sclerosis complex, Peutz-Jeghers syndrome, Cowden disease, Proteus syndrome, tissue/organ hypertrophy including cardiac hypertrophy, etc., is critically required. The findings of the present invention may be the first step toward attainment of such knowledge. This may be supported by the determination of Rheb-mediated regulation of the mTOR pathway. Interaction of PLD2 with Rheb is stabilized in nutrient-rich condition. Also, interaction of PLD2 with raptor is stabilized in nutrient-rich condition. It may be speculated that these two interactions are a regulatory point for nutrient-induced mTOR activation.

Identification of molecular mechanism may provide an important understanding how nutrient impinges on the mTOR complex.

Enhancement of PA production has been reported in various cancer tissues and tumors including prostate cancer and breast cancer. In most cases, this is correlated with overexpression of PLD. However, the mechanism how this is related with tumorigenesis has not been suggested. Our identification of the role of PLD2 in the mTOR signaling suggests the potential molecular mechanism for PLD2-mediated tumorigenesis.

The mTOR signaling has been recognized as an important regulator for metabolic diseases. Insulin receptor substrate-1 (IRS-1) is a newly identified effector for the mTOR signaling. Malfunction of IRS-1 through serine phosphorylation that is mediated by mislocalization and degradation of IRS-1 uncouples normal insulin signaling such as insulin-induced glucose uptake. Uncontrolled regulation of the mTOR signaling by PLD2 and Rheb might be related with mTOR-dependent IRS-1 malfunction.

Taken together, the identification of PLD2 and Rheb as a mediator of mTOR signaling suggests that PLD2 is an important molecular link for nutrient-regulated mTOR signaling, and thereby it presents a novel regulatory point that can be targeted for the treatment of metabolic diseases.

The present invention is further explained in more detail with reference to the following examples. These examples, however, should not be interpreted as limiting the scope of the present invention in any manner.

EXAMPLE 1

Example 1: Preparation of Materials.

The enhanced chemiluminescence kit, glutathione-Sepharose 4B and dipalmitoylphosphatidyl [*methyl*-^{3}H]choline were purchased from Amersham Biosciences. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA/M/G were from Kirkegaard & Perry Laboratories, Inc. (Gainthersburg, MD). Polyclonal antibody was raised against PLD as previously described in "Park,

J.B. *et al.* Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by alpha-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* **275**, 21295-21301 (2000)" and "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001)", which are hereby incorporated by reference.

Antibodies against mTOR, pS6K1 (pThr 389), S6K1 and rapamycin were from Cell Signaling Technology (Beverly, MA). Antibody for Rheb was from SantaCruz Biotechnology (Santa Cruz, CA). Polyclonal raptor antibody was a generously gift from Dr. Do-Hyung Kim (University of Minnesota). Protein A-Sepharose was from RepliGen (Needham, MA) . CHAPS, leucine and leucine-free RPMI-1640 media were from Sigma (St.Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and LipofectAMINE were from Invitrogen (Carlsbad, CA). C-6 phosphatidic acid was from Avanti (Alabaster, AL) and recombinant 4EBP1 was purchased from Stratagene (La Jolla, CA).

Cells and vectors used the following examples were obtained from Invitrogen, unless differently mentioned.

Example 2: Preparation of Plasmids

Mammalian expression vectors for $PLD1^{wt}$, $PLD2^{wt}$, $PLD2^{\Delta N184}$, $PLD2^{\Delta N308}$, $PLD2^{K758R}$ and EGFP-$PLD2^{wt}$ and bacterial expression vectors for GST-tagged PLD2 fragments were used as described previously in "Park, J.B. *et al.* Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by alpha-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* **275**, 21295-21301 (2000)" and "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001)", which are hereby incorporated by reference.

Expression vectors for HA-$mTOR^{wt}$, myc-$mTOR^{wt}$, myc-$raptor^{wt}$, HA-$raptor^{wt}$ and HA-$raptor^{194YDC/AAAmt}$ were gifts from Dr. David M. Sabatini (MIT) (Kim, D.H., *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002), which is hereby incorporated by reference). Mammalian expression vectors for Rheb and bacterial expression vectors for GST-Rheb, GST-Rap1, and GST-R-Ras were kindly provided by Dr. Ariel F. Castro (Indiana University, USA). To introduce the TOS-motif

mutation in PLD2, pCDNA3.1(+)/PLD2 containing wild type PLD2 was PCR amplified using the following oligomers; sense (5'GGC CGA GAC CAA GTT TGT TAT CGC3'; SEQ ID NO: 6), antisense (F265A:5'CCA TCG ATC CGC ACG CCG TGC CGT GCC TCC GTG CTC CTT TTC CCC ACT TGC ACC TCA GCG CCA GG3' ; SEQ ID NO: 7), antisense (E266R:5'CCA TCG ATC CGC ACG CCG TGC CGT GCC TCC GTG CTC CTT TTC CCC ACT TGC ACC CTA AAG CCA GG3' ; SEQ ID NO: 8).

DNA fragments generated by PCR and pCDNA3.1(+)/PLD2(WT) were treated with XhoI and Cla I. His-Rheb was generated by using a standard PCR cloning strategy using myc-Rheb as a template. PCR fragments of Rheb were subcloned in frame into the BamHI/EcoRI sites of pRSET-B vector. All mutant constructs were verified by DNA sequencing. Mammalian expression vectors for myc-S6K1, myc-4EBP1 and HA-raptor fragments (HA-raptor$^{1-646}$, HA-raptor$^{1020-1335}$, HA-raptor$^{647-1335}$, HA-raptor$^{647-1019}$) were generous gifts from Dr. David M. Sabatini (MIT). N-terminal deleted PLD2 fragments were as described previously in "Park, J.B. *et al.* Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by alpha-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* **275**, 21295-21301 (2000)", which is hereby incorporated by reference. GST-fusion PLD2 fragments used for bacterial expression were prepared as previously described in "Park, J.B. *et al.* Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by alpha-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* **275**, 21295-21301 (2000)" and "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001)", which are hereby incorporated by reference.

Example 3: RNA interference

Pairs of 21-nucleotide sense and antisense RNA oligomers were synthesized and annealed by Dharmacon Research, Inc. (Lafayette, CO) as described in "Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291", which is hereby incorporated by reference.

The oligonucleotides used for PLD2 were: sense, 5'-AAG AGG UGG CUG

GUG GUG AAG-3' (SEQ ID NO: 9) and antisense, 5'-CUU CAC CAC CAG CCA CCU CUU-3' (SEQ ID NO: 10), which correspond to human PLD2 coding nucleotides 703-723. All siRNA sequences were subjected to BLAST in the NCBI database and complete matches were only found for PLD2 sequences. Luciferase GL2 duplex was purchased from Dharmacon Research, Inc. and was used as a negative control. The shRNA-encoding lentiviral plasmid was constructed to target the human Rheb mRNA sequence of 5'- GAGGACACTGGGAATATATTC -3' (SEQ ID NO: 11) using the pLKO vector29.

For add-back experiment for PLD2 silencing, three residues of human PLD2 cDNA (nucleotides 703-723 of PLD2; AAGAGGTGGCTGGTGGTGAAG, SEQ ID NO: 12) are substituted to AAGAGATGGCTAGTAGTGAAG for addback mutants of $PLD2^{wt}$. This mutation is silencing mutations. This gene is subcloned into mammalian expression vector pcDNA3.1 (Invitrogen) and digested with restriction enzymes KpnI and XbaI. These mutations are confirmed through nucleotide sequence analysis.

Example 4: Cell culture and plasmid/siRNA transfection

COS7 cells (ATCC, CRL-1651) were maintained in a 5% CO_2 humidified atmosphere at 37℃ and fed DMEM supplemented with 10% bovine calf serum (HyClone). HEK293 cells (ATCC: CRL-1573) were fed DMEM supplemented with 10% fetal bovine serum (HyClone). Cells grown on tissue culture dishes were transiently transfected using LipofectAMINE, as described in "Park, J.B. *et al.* Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by alpha-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* 275, 21295-21301 (2000)" and "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* 276, 28252-28260 (2001)", which are hereby incorporated by reference. Cells were allowed to express the recombinant proteins for 24hr after transfection and then deprived of serum for additional 24hr. The cells were then subjected to leucine deprivation, co-immunoprecipitation, or GST pull-down analysis. Leucine deprivation was performed as previously described 10 with minor modification. Briefly, cells were deprived of serum first for 24 hrs and then subjected to leucine deprivation in leucine-free RPMI 1640 medium.

Example 5: Co-immunoprecipitation.

After harvesting COS7 cells, total extracts were prepared by brief sonication in ice-cold lysis buffer (40mM HEPES pH7.5, 120mM NaCl, 1mM EDTA, 10mM pyrophosphate, 10mM glycerophosphate, 50mM NaF, 1.5mM Na_3VO_4, 0.5% CHAPS, 1mM PMSF, protease inhibitor cocktails). Clarified extracts were mixed with 2 μg of the respective antibodies. Then protein A-Sepharose beads were added to isolate the antibody complex. After four washings with lysis buffer, the final immunoprecipitates were washed once with wash buffer (50mM HEPES pH7.5, 150mM NaCl), and then subjected to SDS-PAGE using Hyperfilm (Amersham Pharmacia Biotech), nitrocellulose membranes (Watmann), Power supply (Amersham Pharmacia Biotech), Electrophoretic Transfer unit (Hoefer Scientific Instruments), and ECL™ (Amersham Pharmacia Biotech).

Example 6: In vitro binding analysis.

GST fusion proteins (PLD2 fragments, GST-Rheb, GST-Rap1, and GST-R-Ras) expressed in *E.* coli (Invitrogen) were isolated using glutathione-Sepharose 4B beads and then incubated with cell lysates expressing either PLD2 or HA-Rheb^{wt} ectopically. After incubation at 4℃ for 2hr, the beads were washed and then subjected to SDS-PAGE. *E. coli* and COS7 cells were lysed with the lysis buffer used for the co-immunoprecipitation. Hexa-histidine (His6)-tagged PLD2 was purified from detergent extracts of baculovirus-infected Sf9 cells (Invitrogen) by chelating Sepharose affinity column chromatography, as previously described in Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001), which is hereby incorporated by reference. As indicated, recombinant proteins (GST-Rheb, GST-2F3, GST-2F2, and His-Rheb) were further eluted after affinity purification using either GSH or nickel. *In vitro* binding analysis was done in ice-cold lysis buffer (as used for the co-immunoprecipitation but including 10% glycerol).

Example 7: Western blot analysis.

Proteins were separated by SDS-PAGE on 8-16% gradient gels, and the separated proteins were transferred onto nitrocellulose membranes and blocked with TTBS buffer (10mM Tris-HCl, pH 7.6, 150mM NaCl, and 0.05% Tween-20)

containing 5% skimmed milk powder. The SDS-PAGE was performed using Hyperfilm (Amersham Pharmacia Biotech), nitrocellulose membranes (Watmann), Power supply (Amersham Pharmacia Biotech), Electrophoretic Transfer unit (Hoefer Scientific Instruments), and ECL™ (Amersham Pharmacia Biotech). Membranes were then incubated with primary antibody at the concentration recommended by the manufacturer for 4hr at room temperature. Unbound antibody was washed away with TTBS buffer. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 1hr at room temperature, washed five times with TTBS buffer, and developed using an ECL system.

Example 8: In vivo PLD assay.

In vivo PLD activity was assayed by measuring the formation of phosphatidyl-butanol as described in "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001)" and "Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291", which are hereby incorporated by reference.

In brief, cells were loaded with [^{3}H]myristic acid (2μCi/ml) for 8hr and then washed twice with DMEM. Labeled cells were incubated with 0.4% butanol for 10min to measure basal PLD activity. Total lipids were extracted with 1.2 ml of methanol:1M NaCl: chloroform (1:1:1 by volume) and then separated by thin-layer chromatography on silica gel plates. The amount of [^{3}H]phosphatidyl-butanol formed was expressed as a percentage of total [^{3}H]lipid to account for cell labeling efficiency differences.

Example 9: *In vitro* kinase assay for mTOR activity.

Recombinant myc-mTOR was expressed with the indicated proteins and then immunoprecipitated using anti-myc antibody, as previously described in "Lee, S. *et al.* Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276**, 28252-28260 (2001)" and "Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.*

18 (2006) 2283-2291", which are hereby incorporated by reference. Recombinant 4EBP1 (Stratagen) was used as a substrate for *in vitro* kinase assays. Activities were measured using anti-phospho-4EBP1 antibody (phosphor-37/46). The kinase assay was performed by mixing buffer containing 25mM Hepes pH7.4, 50mM KCl, 10mM $MgCl_2$, 4mM $MnCl_2$, 20% glycerol, 2mM DTT, 0.1mM ATP, 1μg 4EBP1 with the indicated immunoprecipitates and then incubated at 30℃ for 15min.

Example 10: Examination of Interrelation between PLD2 and Rheb

The investigation of the present invention was initiated to test whether functional and/or physical connections between PLD and Rheb exist because no report to date has addressed their interrelationships. Also, it has been reported that Rheb activates mTOR via unknown mechanism.

To examine the potential involvement of PLD in Rheb-mediated mTOR activation, each PLD isozyme (mammals have two, PLD1 and PLD2, which share about 50% identity) was silenced by specific siRNA. HA-$Rheb^{Q64L}$ was expressed in HEK293 cells with the indicated siRNAs. Resulting lysates were immunoblotted with the antibodies in Fig. 1. The obtained results were shown in Fig. 1, showing that knock-down of PLD2 specifically reduced the phosphorylations of S6K1 and 4EBP1, which have been well-defined mTOR substrates. It is likely that the involvement of PLD2 in Rheb-induced mTOR activation is specific since the phosphorylation of extracellular signal-regulated protein kinase (ERK) induced by Rheb was not modulated by knock-down of either PLD1 or PLD2 or both.

As an opposite direction, to determine whether Rheb is required for PA generation, the effect of Rheb on the activities of PLD isozymes was tested. PLD2 or PLD1 was transfected into COS7 cells with shRNA against Rheb. After 48hr, cells were deprived of serum for 20hr, labeled with [^{3}H]myristic acid for 4hr, and subjected to PLD assay, as described in Examples 1-8. Posphatidyl-butanol formation (PBt formation) were measured and shown in Fig. 2. Silencing of Rheb was verified by Western blot analysis and presented within graph of Fig. 2 indicated as shRheb. The result in Fig. 2 reveals that effect of Rheb was restricted to PLD2 by Rheb silencing, suggesting that Rheb is required for PLD2 specifically. These results demonstrate that both PLD2 and Rheb are required for mTOR activation.

Example 11: Direction of Interaction between PLD2 and Rheb

The physical connection between PLD2 and Rheb to support their functional connection was tested. HA-Rheb^{wt} with either PLD2 or PLD1 in COS7 cells was overexpressed. The levels of PLD isozymes in HA-immunoprecipitates were examined. HA-Rheb^{wt} was transfected with PLD2 or PLD1 and resulting lysates were immunoprecipitated with anti-HA antibody. Western blot analysis was performed as described above to detect bound PLD isozymes in HA-immunoprecipitates. The obtained results were shown in Fig. 3 (IP; immunoprecipitation, T.Lys.; Total lysates). As shown in Fig. 5, PLD2 was found, but not PLD1, in HA immunoprecipitates. The GTP- or GDP- forms of Rheb did not show any preference in terms of interaction with PLD2 in cells or *in vitro*.

The interaction between PLD2 and Rheb was further tested by GST-pull down analysis as described in Example 6. GST-fusion proteins (GST-Rheb, GST-Rap1 and GST-R-Ras) were expressed and then used for GST-pull down analysis with PLD2-expressing cell lysates. The obtained results were shown in Fig. 4, wherein GST-fusion proteins are denoted by asterisks. Fig. 4 shows that PLD2 interacted with GST-Rheb but not with GST-Ras or GST-Rap1. This interaction was specific to PLD2, as PLD1 was not detected in the same pull-down experiments.

To understand how Rheb interacts with PLD2, a series of truncated PLD2 mutants were prepared and their abilities to bind Rheb were tested by GST-pull down analysis as described in Example 6. The obtained results were shown in Fig. 5, wherein the upper panel is a schematic view of the PLD2 fragments used, and the lower panel shows the result of GST-pull down analysis. As shown in Fig. 5, the Rheb docking site on PLD2 was mapped to a region encompassing amino acid 476-612 of full-length Rheb.

To reveal whether their interaction is direct, purified PLD2 and Rheb were reconstituted for *in vitro* binding analysis as described above. Recombinant PLD2 and His-Rheb were expressed and purified from Sf9 cells and *E.coli*, respectively. In addition, GST, GST-2F3 (amino acids 476-612 of PLD2), and GST-2F2 (amino acids 315-475 of PLD2) were expressed and purified from *E.coli* using GSH-Sepharose beads. The purified GST-fusion proteins (40nM each) were mixed with protein mixture containing PLD2 and His-Rheb and then anti-PLD antibody and

protein A-agarose bead were added to precipitate PLD2 from the protein mixture. Bound proteins were detected by Western blotting as described above. The obtained results were shown in Fig. 6. As shown in Fig. 6, their interaction was direct through amino acid 476-612 (designated as 2F3 in Fig. 5) of PLD2. Taken together, Rheb and PLD2 are connected both physically and functionally, and their interaction results in PA generation.

Example 12: Investigation of PLD2 mediates Rheb binding to the mTOR complex and its effect on Rheb activation of mTOR

Based on the inventors' previous finding that the Rheb/PLD2 complex is related to the PLD2/raptor/mTOR complex (Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291, which is hereby incorporated by reference) and the results obtained above, this example examined whether mTOR complex contains raptor, PLD2, and Rheb.

COS7 cell lysates were prepared using different lysis conditions, CHAPS and Triton X-100, and then subjected to co-IP against anti-mTOR antibody described above. The obtained results were shown in Fig. 7. As shown in Fig. 7, raptor, PLD2 and Rheb were found in the mTOR complex.

Rheb was found to bind raptor complex, and this interaction was enhanced by PLD2 in a TOS motif-dependent manner as shown in Fig. 8. HA-Rheb^{Q64L} was transfected with the indicated PLD2 mutants into COS7 cells and the resulting lysates were immunoprecipitated with anti-HA antibody. Levels of bound raptor and PLD2 were detected by Western blotting. The obtained results shown in Fig. 8 suggest the importance of PLD2 as a molecular bridge between Rheb and mTOR complex.

The enhanced binding of Rheb with mTOR complex was also confirmed by *in vitro* binding analysis with purified mTOR immunoprecipitates as described above and the results were shown Fig. 9. After transfection of HA-mTOR^{wt} and myc-raptor^{wt}, the mTOR/raptor complex was immunoprecipitated with anti-HA antibody. The resulting immunoprecipitate was further incubated for *in vitro* binding analysis with purified PLD2 and GST-Rheb (40nM) as indicated, in the presence of 100μM

GTPγS. After incubation for 2hr, immunoprecipitates were rewashed and Western blotted.

The importance of PLD2 as a physical link in mTOR signaling was further highlighted by PLD silencing followed by PA treatment, which was shown to increase mTOR activity in cells as reported in the inventors' previous report (Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291, which is hereby incorporated by reference). It has been reported that, interestingly, PA could no longer activate mTOR pathway when PLD2 expression was lowered. However, PA could rescue mTOR activity when PLD1 expression was lowered, suggesting the importance of PLD2 itself to activate mTOR kinase activity. The importance of the link between PLD2 and Rheb was tested using small fragments, 2F3 and 2F2, derived from the Rheb binding region of PLD2. After transfecting HA-Rheb^{Q64L} with GFP-2F3 or GFP-2F2, cells were deprived of serum and then co-IP analyses were performed using anti-HA antibody as described above. The obtained results were shown in Fig. 10. As shown in Fig. 10 as well as Fig. 5, Rheb interaction with PLD2 in cells was abrogated by GFP-2F3, but not by GFP-2F2. This is well correlated with decreased S6K1 phosphorylation by GFP-2F3 overexpression in Rheb-overexpressed cells (Fig. 10).

To test whether Rheb-induced mTOR kinase activity is also downregulated by GFP-2F3 overexpression, the following test was performed. After transfecting HA-Rheb^{Q64L}, GFP-2F3 and myc-mTOR^{wt}, *in vitro* mTOR kinase assays were performed using anti-myc immunoprecipitates as described above. The results were shown in Fig. 11. As confirmed in Fig. 11, Rheb-induced mTOR kinase activity was also downregulated by GFP-2F3 overexpression, suggesting the importance of Rheb/PLD2 binding for Rheb-induced mTOR activation. This raised the possibility that the enzymatic activation of PLD2 together with its physical link is important for the Rheb activation of mTOR.

Example 13: Role of PA in Rheb activity to activate mTOR kinase activity

The mechanism by which PA activates mTOR in cells was not revealed by an earlier study (Kim, D.H., *et al.* mTOR interacts with raptor to form a nutrient-

sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002), which incorporated herein as a reference). Only a conformational change of mTOR or its specific recruitment of an upstream regulator was suggested.

This example examined the speculation that PA could increase Rheb/mTOR binding and activate mTOR through PA-dependent conformational change of mTOR. After transfection of HA-Rheb^{Q64L}, cells were deprived of serum for 24 hr, then treated with 1-butanol (1-BtOH, 0.4%), t-butanol (t-BtOH, 0.4%), phosphatidic acid (C-6 PA, 100uM), and rapamycin (20nM), respectively. The obtained results were shown in Fig. 12. The speculation was further forced because the results shown in Fig. 12 shows that PA is essentially required for Rheb to activate the mTOR signaling, as verified in rescue experiment from 1-butanol-treated cells by PA. Also, it was observed that the interaction between mTOR and Rheb was attenuated in 1-butanol-treated cells, which suggested the importance of PA production for their interaction.

mTOR/raptor/PLD2 was purified by immunoprecipitation, and then used as *in vitro* bait for purified GST-Rheb together with PA. HA-mTOR, myc-raptor, and PLD2 were transfected and resulting lysates were immunoprecipitated with anti-HA antibody. The resulting immunoprecipitates were used for *in vitro* binding analysis with purified GST-Rheb (40nM), PA (10μM), GDPβS (100μM), or GTPγS (100μM). The obtained results were shown in Fig. 13. As shown in Fig. 13, PA increased GST-Rheb binding to mTOR complex in the presence of GTPγS.

This enhanced binding was correlated with mTOR activation both *in vitro* and *in vivo*, as shown in Figs. 14 and 15. Myc-mTOR, HA-raptor and PLD2 were transfected into HEK293 cells and resulting lysates were immunoprecipitated with anti-myc antibody. The resulting immunoprecipitate was used for *in vitro* mTOR kinase assay. The obtained results were shown in Fig. 14 (IVK: in vitro kinase assay). HA-mTOR^{wt} and myc-Rheb^{wt} or myc-Rheb^{D60I} were transfected into COS7 cells and allowed to express for 24hr. After 24 serum starvation, 100 μM PA was treated to cells and the resulting lysates were subjected to *in vitro* kinase assay for mTOR. The obtained results were shown in Fig. 15.

Taken together, the above results suggest that PLD2 provides a physical link between Rheb and the mTOR complex, and that, as a result, the PA generated in the proximity of the mTOR complex increases Rheb/mTOR complex binding, which result in mTOR activation.

Example 14: Investigation of nutrient dependent PLD2 activation

Previously, the inventors suggested that both the PLD2/raptor interaction and the enzymatic activity of PLD2 are required for mTOR pathway stimulation (Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Kim, J.H., Jang, S.K., Suh, P.G., Ryu, S.H. PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. *Cell. Signal.* 18 (2006) 2283-2291, which is hereby incorporated by reference). Again, these relations suggest that PLD2 is a new binding protein that has physical and functional connections with the mTOR complex.

Until now, the function of PLD2 in nutrient signaling has not been proposed. To this end, the importance of PLD2 in nutrient signaling was tested. The stimulatory effect of leucine on S6K1 was significantly reduced by 1-butanol. Based on the result, PLD2 activity in response to nutrient levels was checked. $PLD2^{wt}$ was transfected and allowed to express for 24hr. After serum-deprivation for 16hr and labeling with [^{3}H]myristic acid (2(Ci/ml) for 8hr, cells were treated with rapamycin (20nM), D-PBS, and leucine-free media (biowhittaker). After 45min, same treatments including leucine-added media were added with 0.4% 1-butanol to measure PBt formation. The obtained results were shown in Fig. 16, indicating that PLD2 activity was reversibly regulated by amino acid levels, especially by leucine levels.

To reveal the relationship between PLD2 activity and complex formation, the interaction between PLD2 and mTOR was tested. mTOR inhibition by rapamycin, which is a mTOR specific inhibitor, had no effect on the interaction between PLD2 and mTOR. However, the interaction between endogenous PLD2 and endogenous mTOR complex was increased by leucine treatment in leucine-deprived COS7 and HEK293 cells as shown Fig. 17. The results in Fig. 17 were obtained by lysing confluent COS7 and HEK293 cells and immunoprecipitating with anti-mTOR antibody after treating leucine-deprived cells with leucine.

Either HA-$raptor^{wt}$/$PLD2^{wt}$ or HA-$Rheb^{wt}$/$PLD2^{wt}$ was transfected into HEK293 cells, and the cells were subjected to leucine deprivation. Co-IP after leucine treatment was followed by Western blot analysis. The results were shown in Figs. 18 (for HA-$raptor^{wt}$/$PLD2^{wt}$) and 19 (HA-$Rheb^{wt}$/$PLD2^{wt}$). The increase by

leucine was attributed to enhanced binding between PLD2 and raptor, as shown in Fig. 18. Importantly, the interaction between Rheb and PLD2 was also regulated by nutrient levels such that this interaction was stabilized under high nutrient conditions and weakened under low nutrient conditions, which allowed a nutrient-dependent mTOR complex composed of mTOR, raptor, PLD2, and Rheb to be identified, as shown in Fig. 19. These results are well correlated with reversible regulation of PLD2 activity against the level of leucine.

Example 15: Essential role of PLD2/Rheb interaction in nutrient (leucine) signaling

After transfecting PLD2 siRNA, cells were deprived of leucine for 45min. The cells were treated with various concentration of leucine (0-100μM) for 15 min and the resulting lysates were used for Western blot analysis. The obtained results were shown in Fig. 20. As shown in Fig. 20, it was found that PLD2 silencing completely downregulated the effect of leucine on S6K1 phosphorylation.

Further, HA-Rheb^{Q64L} was transfected with either GFP-2F3 or GFP-2F2 and the resulting cells were subjected to leucine deprivation for 45 min. After leucine (100μM) treatment for 15 min, the cell lysates were used for Western blot analysis. The obtained results were shown in Fig. 21, showing that the effect of leucine on S6K1 phosphorylation was specifically downregulated when GFP-2F3 was overexpressed. Such results suggest the importance of Rheb/PLD2 binding on leucine-induced mTOR signaling.

This increased mTOR activity was found to be well correlated with the regulation of cell growth, the control of cell size. After transfecting the PLD2 mutants as indicated in Fig. 22, cells were cultured to confluence. Cell sizes were determined after replating these cells for 48hr, using a Multisizer 3 (Beckman Coulter), as described in "Kim, D.H., *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002)", which is hereby incorporated by reference. The obtained results were shown in Fig. 22, showing that PLD2 overexpression increased cell size in a TOS-motif dependent manner. Such results demonstrate the functional role of PLD2 in mTOR signaling for cell growth control, which is achieved via mTOR/raptor/PLD2/Rheb complex formation.

WHAT IS CLAIMED IS:

1. A method of regulating mammalian target-of-rapamycin (mTOR) activity by regulating one or more steps among the following steps:

1) binding of Ras homolog enriched in brain (Rheb) to mTOR through phospholipase D (PLD) 2 by interaction between PLD2 and Rheb;

2) moving PA produced from PLD2 near to mTOR to increase the level of phosphatidic acid (PA) near mTOR; and

3) activating the mTOR kinase activity by binding of Rheb to mTOR due to the increased level of PA near mTOR.

2. The method according to Claim 1, wherein regulation of the interaction between PLD2 and Rheb is conducted by modifying a Rheb binding site of PLD2, a PLD2 binding site of Rheb, or both of them, and the Rheb binding site of PLD2 comprises the amino acid residues from position 476 to position 612 of full-length PLD2, resulting in inhibiting the mTOR activity.

3. The method according to Claim 2, wherein the regulation of the interaction between PLD2 and Rheb is conducted by deleting one or more amino acids selected from the amino acid residues from position 476 to position 612 of full-length PLD2, resulting in inhibiting the mTOR activity.

4. The method according to Claim 1, wherein the regulation of binding of Rheb to mTOR through PLD2 is conducted by removing phosphatidic acid (PA) or GTPγS, resulting in inhibiting the mTOR activity.

5. The method according to Claim 1, wherein the regulation of interaction between PLD2 and Rheb is conducted by decreasing the level of leucine, resulting in inhibiting the mTOR activity.

6. A method of screening inhibitors of mTOR activity, comprising the steps of:

contacting a candidate compound to a sample cell;

examining the interaction between PLD2 and Rheb by which Rheb is capable of binding to mTOR through PLD2; and

determining the compound as an inhibitor of mTOR activity when the level of the interaction between PLD2 and Rheb decreases compared with that in other sample cells without contacting with the compound.

7. The method according to Claim 6, wherein the sample cell is selected from the group consisting of a human embryonic kidney, a human epithelial ovarian cancer cell, COS7 cell, a human cervical cancer cell, a human colon cancer cell, a human breast cancer cell, a human hepatoma, a human breast cancer cell, and a human T cell leukemia.

8. The method according to Claim 6, wherein the inhibitor of mTOR activity is used as an agent of treating a mTOR-related metabolic disease.

9. The method according to Claim 8, wherein the mTOR-related metabolic disease is one or more selected from the group consisting of cancer, diabetes, obesity, hamartoma syndromes and tissue/organ hypertrophy.

10. A method of treating mTOR-related metabolic disease, by inhibiting one or more steps among the steps of:

1) binding of Ras homolog enriched in brain (Rheb) to mTOR through phospholipase D (PLD) 2 by interaction between PLD2 and Rheb;

2) moving PA produced from PLD2 near to mTOR to increase the level of phosphatidic acid (PA) near mTOR; and

3) activating the mTOR kinase activity by binding of Rheb to mTOR due to the increased level of PA near mTOR.

11. The method according to Claim 10, wherein the mTOR-related metabolic disease is one or more selected from the group consisting of cancer, diabetes, obesity, hamartoma syndromes and tissue/organ hypertrophy.

12. The method according to Claim 10, wherein the inhibition of the

interaction between PLD2 and Rheb is conducted by modifying a Rheb binding site of PLD2, a PLD2 binding site of Rheb, or both of them, and the Rheb binding site of PLD2 comprises the amino acid residues from position 476 to position 612 of full-length PLD2.

13. The method according to Claim 12, wherein the inhibition of the interaction between PLD2 and Rheb is conducted by deleting one or more amino acids selected from the amino acid residues from position 476 to position 612 of full-length PLD2.

14. The method according to Claim 10, wherein the inhibition of the binding of Rheb to mTOR through PLD2 is conducted by removing phosphatidic acid (PA) or GTPγS.

15. The method according to Claim 10, wherein the inhibition of the interaction between PLD2 and Rheb is conducted by removing leucine.

16. A composition for treating a mTOR-related metabolic disease, containing an effective amount of a mTOR inhibitor as an active ingredient, wherein the mTOR inhibitor has the activity to inhibits one or more steps among the steps of:

1) binding of Ras homolog enriched in brain (Rheb) to mTOR through phospholipase D (PLD) 2 by interaction between PLD2 and Rheb;

2) moving PA produced from PLD2 near to mTOR to increase the level of phosphatidic acid (PA) near mTOR; and

3) activating the mTOR kinase activity by binding of Rheb to mTOR due to the increased level of PA near mTOR.

17. The composition according to Claim 16, wherein the mTOR-related metabolic disease is one or more selected from the group consisting of cancer, diabetes, obesity, hamartoma syndromes and tissue/organ hypertrophy.

18. The composition according to Claim 16, wherein the mTOR inhibition is a material that modifies Rheb binding site of PLD2, a PLD2 binding site

of Rheb, or both of them, and the Rheb binding site of PLD2 comprises the amino acid residues from position 476 to position 612 of full-length PLD2, to inhibit the interaction between PLD2 and Rheb.

19. The composition according to Claim 16, wherein the mTOR inhibitor is a material that decreases the level of phosphatidic acid (PA) or GTPγS.

20. The composition according to Claim 16, wherein the mTOR inhibitor is a material that decreases the level of leucine.

21. A target polypeptide for screening an agent of treating one or more mTOR-related metabolic diseases selected from the group consisting of cancer, diabetes, obesity, hamartoma syndromes and tissue/organ hypertrophy, consisting essentially of the amino acid sequence from position 476 to position 612 of full-length PLD2.

Fig. 1

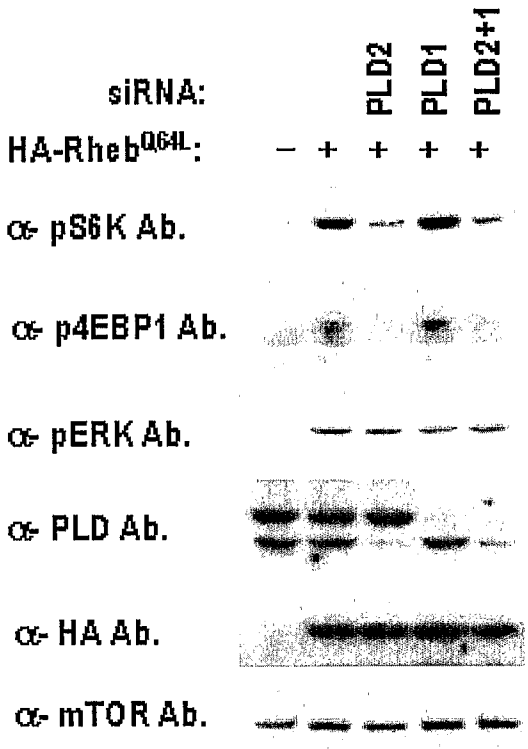

siRNA:
PLD2
PLD1
PLD2+1
HA-Rheb^{Q64L}: – + + + +
α- pS6K Ab.
α- p4EBP1 Ab.
α- pERK Ab.
α- PLD Ab.
α- HA Ab.
α- mTOR Ab.

Fig. 2

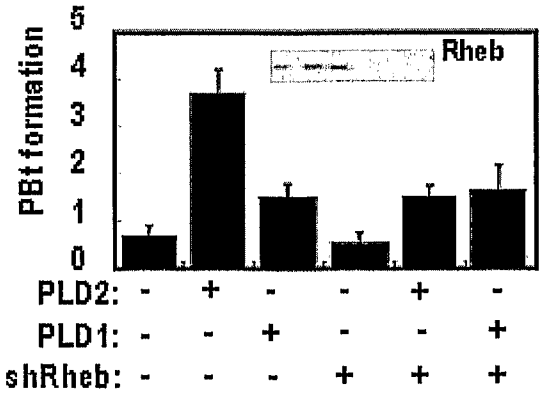

PBt formation
5
4
3
2
1
0
Rheb
PLD2: - + - - + -
PLD1: - - + - - +
shRheb: - - - + + +

Fig. 3

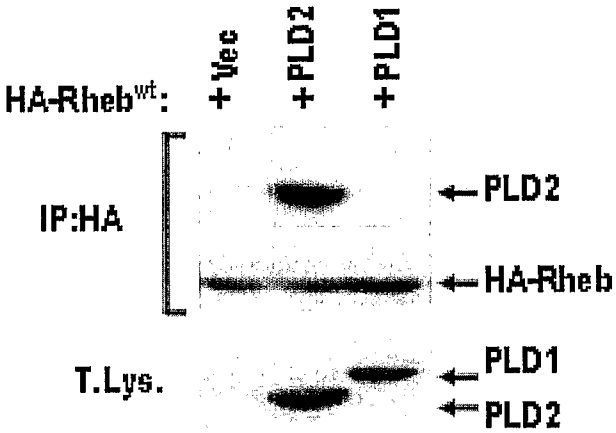

HA-Rheb^{wt}:
+ Vec
+ PLD2
+ PLD1
IP:HA
PLD2
HA-Rheb
T.Lys.
PLD1
PLD2

Fig. 4

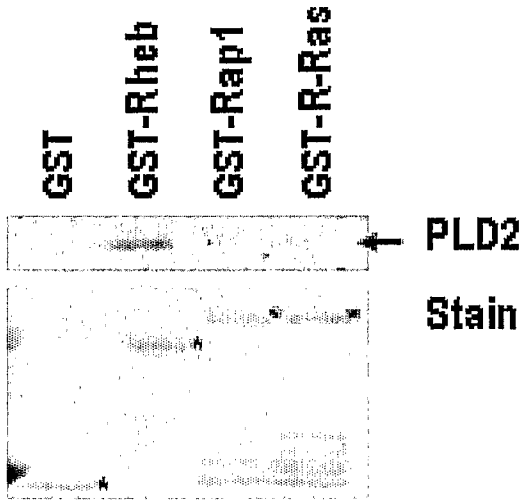

GST
GST-Rheb
GST-Rap1
GST-R-Ras
PLD2
Stain

Fig. 5

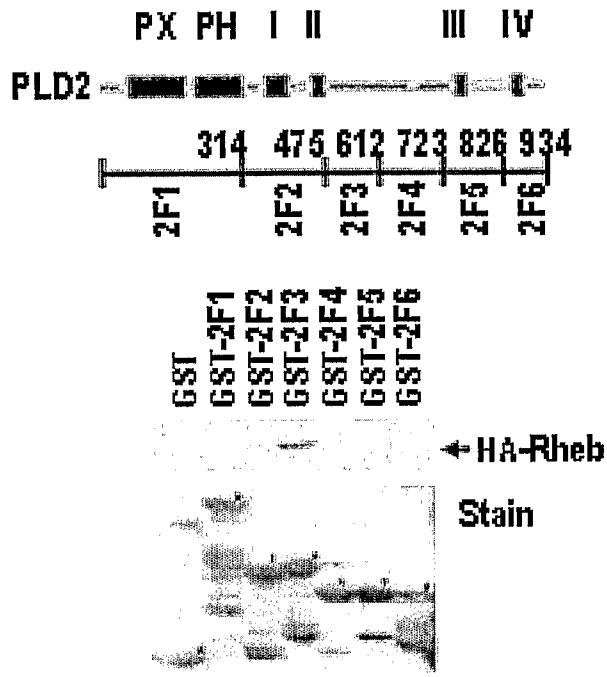

PX PH I II III IV
PLD2
314 475 612 723 826 934
2F1 2F2 2F3 2F4 2F5 2F6
GST GST-2F1 GST-2F2 GST-2F3 GST-2F4 GST-2F5 GST-2F6
HA-Rheb
Stain

Fig. 6

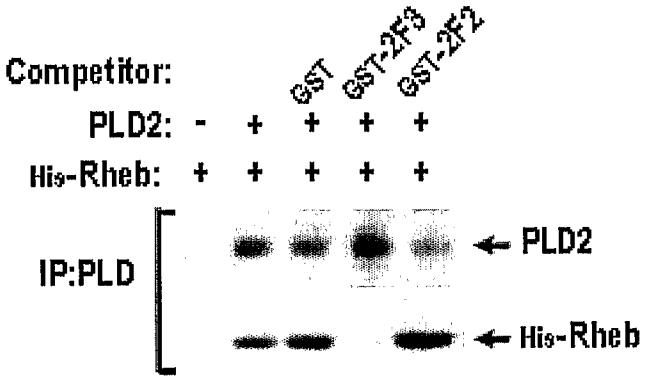

Competitor:
GST
GST-2F3
GST-2F2
PLD2: - + + + +
His-Rheb: + + + + +
IP:PLD
PLD2
His-Rheb

Fig. 7

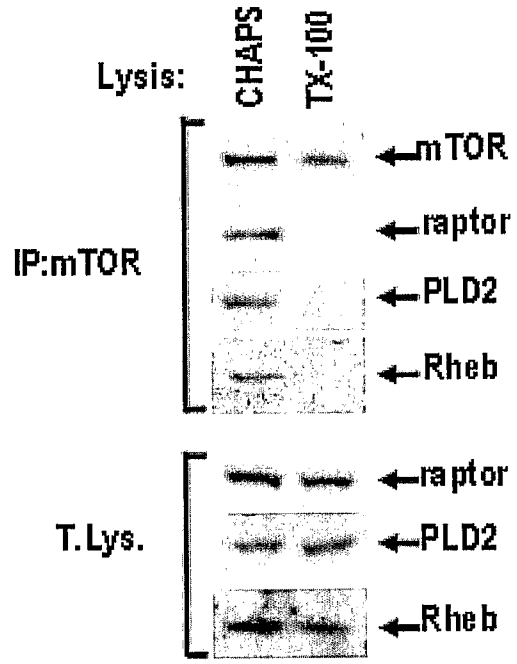

Lysis:
CHAPS
TX-100
IP:mTOR
mTOR
raptor
PLD2
Rheb
T.Lys.
raptor
PLD2
Rheb

Fig. 8

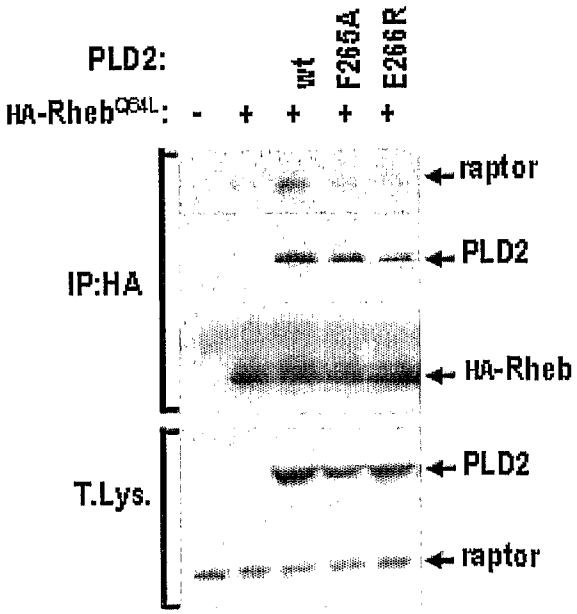

PLD2:
wt
F265A
E266R
HA-Rheb^{Q64L}: - + + + +
IP:HA
raptor
PLD2
HA-Rheb
T.Lys.
PLD2
raptor

Fig. 9

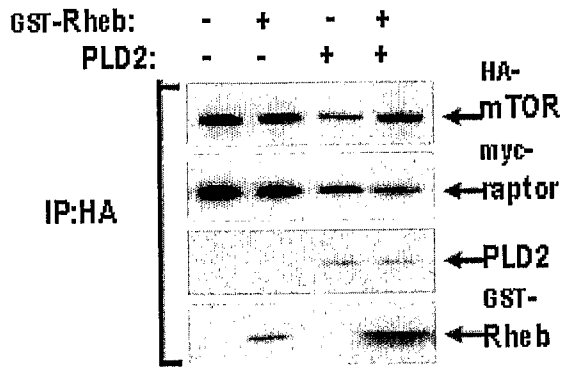

GST-Rheb: - + - +
PLD2: - - + +
IP:HA
HA-mTOR
myc-raptor
PLD2
GST-Rheb

Fig. 10

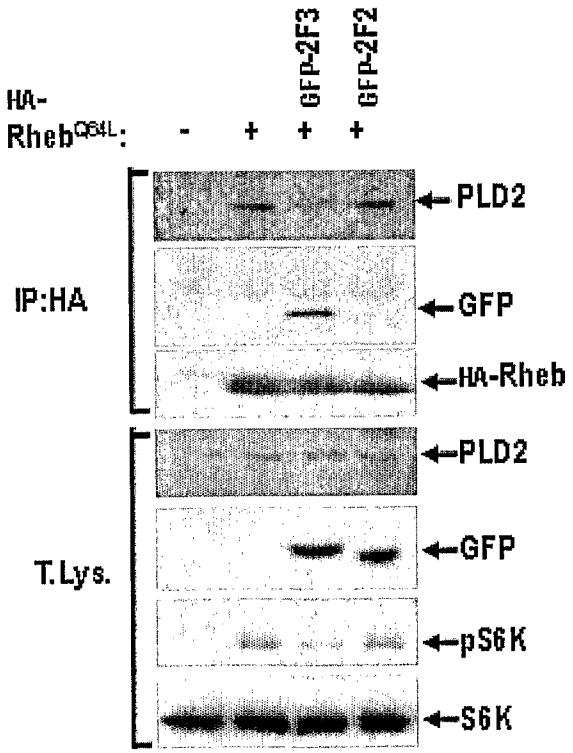

HA-Rheb^{Q64L}: - + + +
GFP-2F3
GFP-2F2
IP:HA
PLD2
GFP
HA-Rheb
T.Lys.
PLD2
GFP
pS6K
S6K

Fig. 11

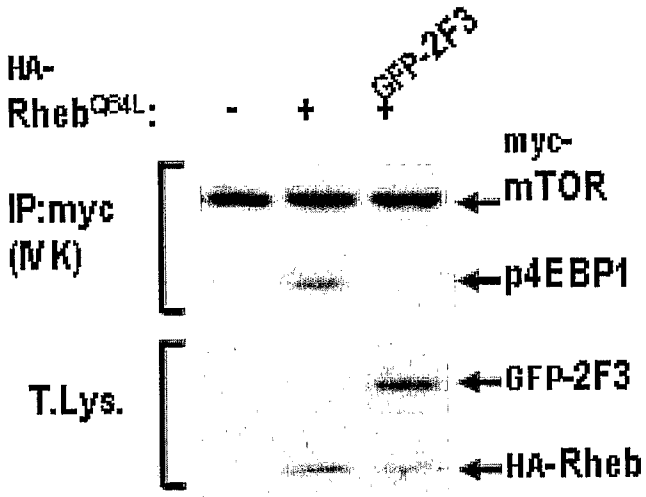

HA-
Rheb^{Q64L}: - + +GFP-2F3
myc-
mTOR
IP:myc
(IVK)
p4EBP1
T.Lys.
GFP-2F3
HA-Rheb

Fig. 12

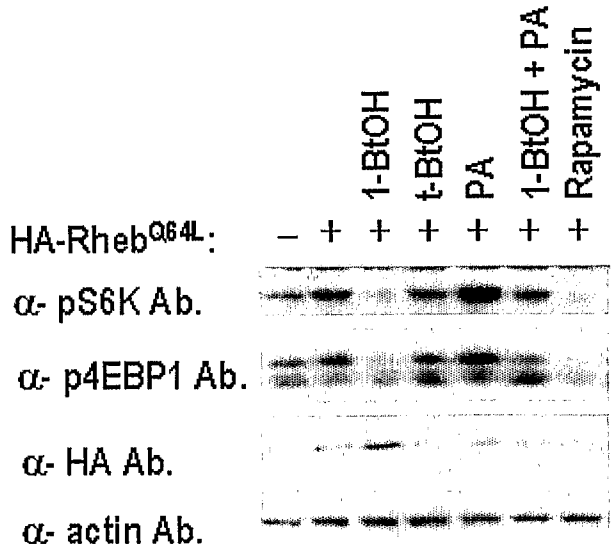

1-BtOH
t-BtOH
PA
1-BtOH + PA
Rapamycin
HA-Rheb^{Q64L}: – + + + + + +
α- pS6K Ab.
α- p4EBP1 Ab.
α- HA Ab.
α- actin Ab.

Fig. 13

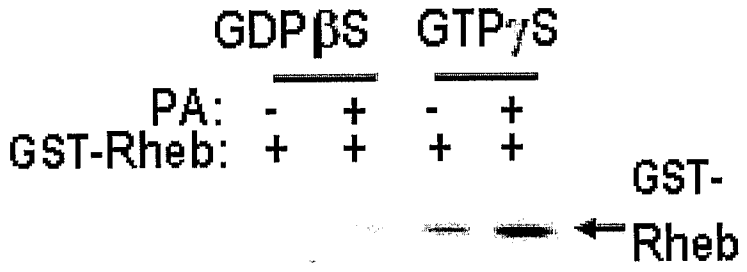

GDPβS
GTPγS
PA: - + - +
GST-Rheb: + + + +
GST-
Rheb

Fig. 14

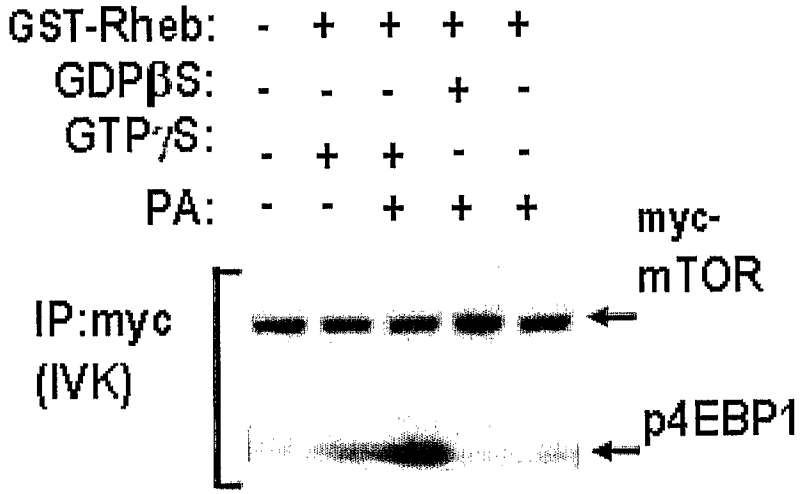

GST-Rheb: - + + + +
GDPβS: - - - + -
GTPγS: - + + - -
PA: - - + + +
myc-
mTOR
IP:myc
(IVK)
p4EBP1

Fig. 15

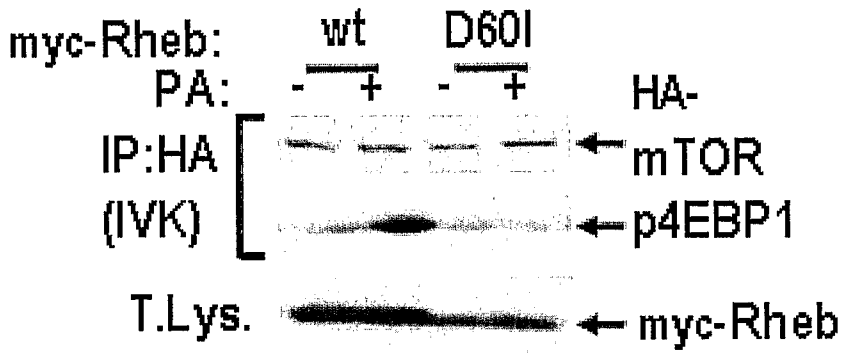

myc-Rheb:
wt
D60I
PA:
- + - +
HA-
IP:HA
mTOR
(IVK)
p4EBP1
T.Lys.
myc-Rheb

Fig. 16

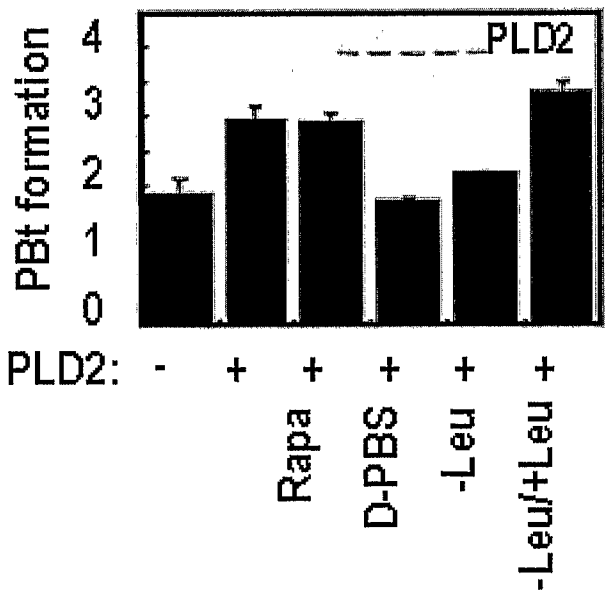

PBt formation
4
3
2
1
0
PLD2
PLD2: - + + + + +
Rapa
D-PBS
-Leu
-Leu/+Leu

Fig. 17

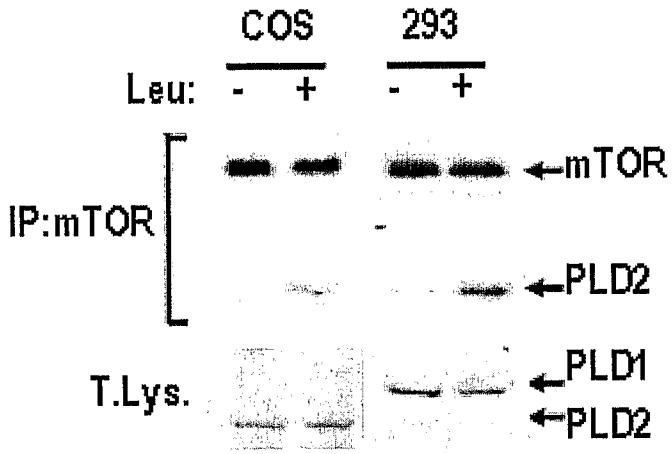

COS
293
Leu: - + - +
IP:mTOR
←mTOR
←PLD2
T.Lys.
←PLD1
←PLD2

Fig. 18

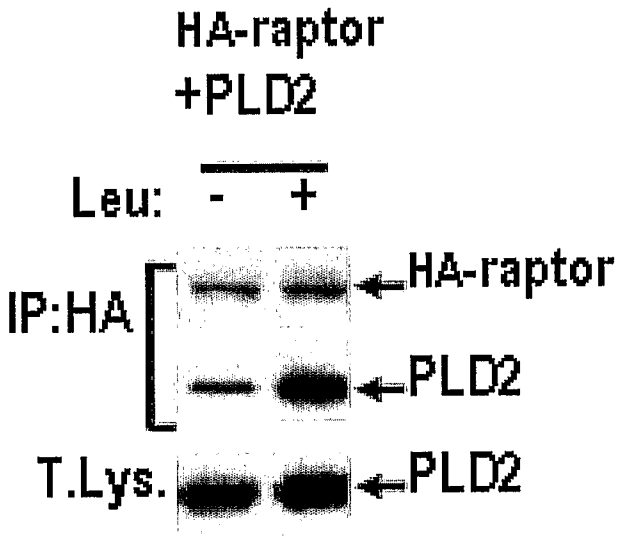

HA-raptor
+PLD2
Leu: - +
IP:HA
←HA-raptor
←PLD2
T.Lys.
←PLD2

Fig. 19

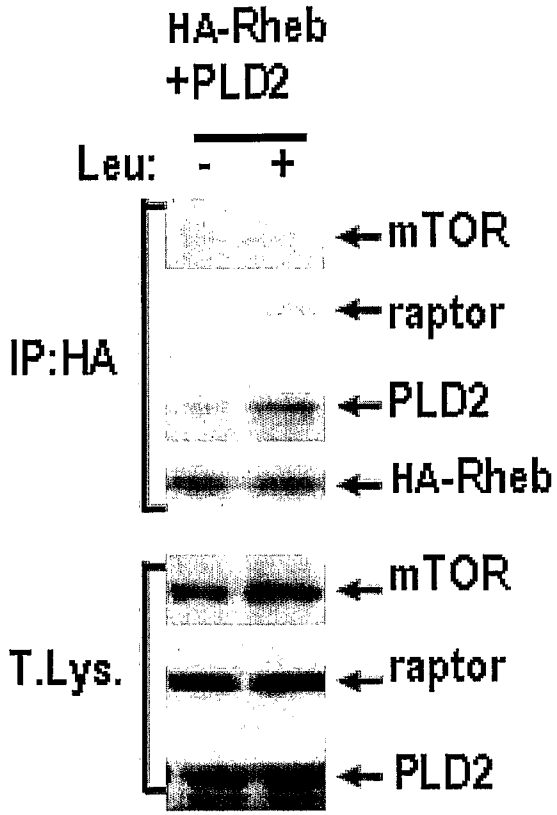

HA-Rheb
+PLD2
Leu: - +
IP:HA
mTOR
raptor
PLD2
HA-Rheb
T.Lys.
mTOR
raptor
PLD2

Fig. 20

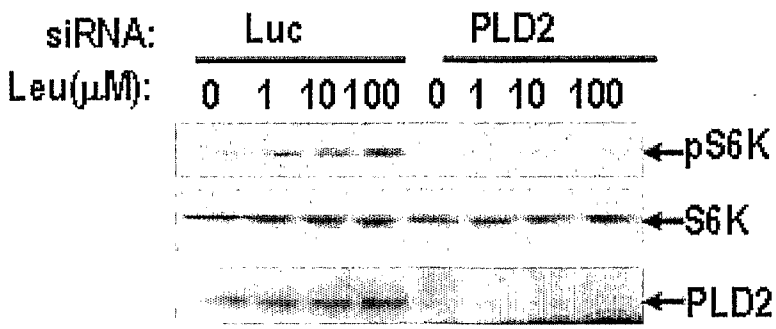

siRNA: Luc PLD2
Leu(μM): 0 1 10 100 0 1 10 100
pS6K
S6K
PLD2

Fig. 21

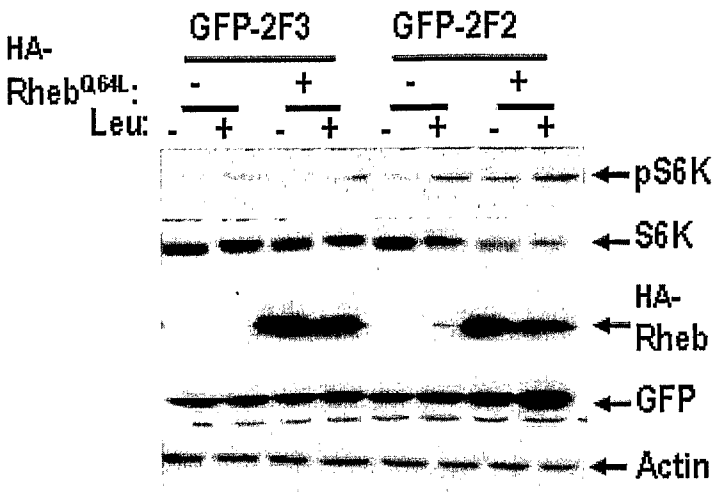

GFP-2F3
GFP-2F2
HA-
Rheb^{Q64L}:
Leu:
pS6K
S6K
HA-
Rheb
GFP
Actin

Fig. 22

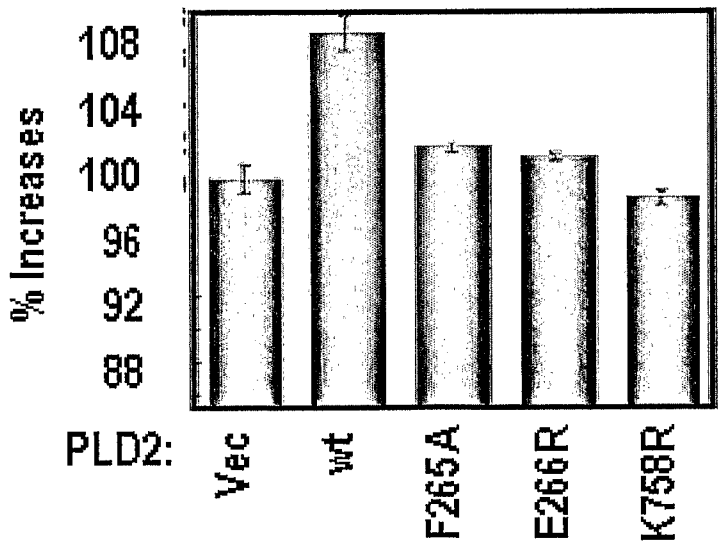
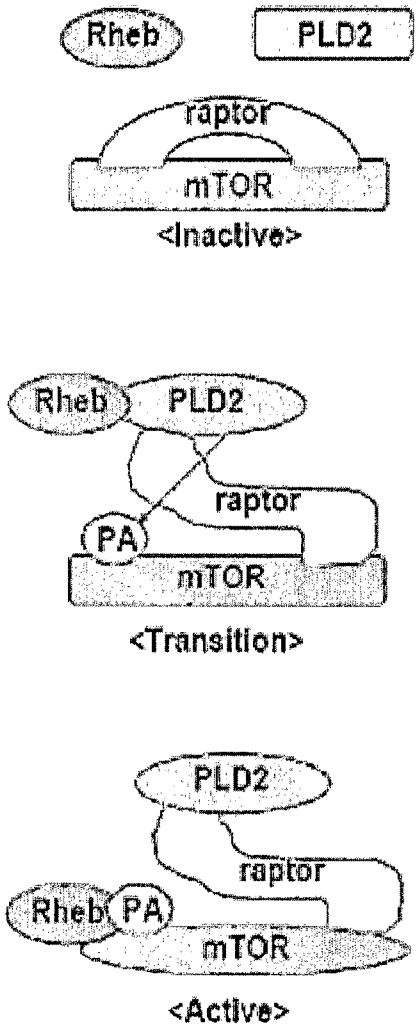

% Increases
108
104
100
96
92
88
PLD2:
Vec
wt
F265A
E266R
K758R

Fig. 23


Rheb
PLD2
raptor
mTOR
<Inactive>
Rheb
PLD2
raptor
PA
mTOR
<Transition>
PLD2
raptor
Rheb
PA
mTOR
<Active>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/003754

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/16(2006.01)i, A61K 38/43(2006.01)i, A61P 35/00(2006.01)i, A61P 3/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K 38/16, 31/436, A61P 3/04, 35/00, C07K 7/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), PubMed, JPO, USPTO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,Y	Sang Hoon Ha et al., 'PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals', Cellular Signalling, online publication on 3 June 2006. See abstract, page 2284-2290	16-21
Y	Xiameng Long et al., 'Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency', The Joural of Biological Chemistry, Vol. 280(25) pp. 23433-23463, 24 June 2005. See abstract, page 23433-23463	16-21
A	WO 2004/026898 A1 (POSTECH FOUNDATION) 1 April 2004. See abstract, page 10-19, claims 1-13	16-21
A	Nissim Hay and Nahum Sonenberg, 'Upstream and downstream of mTOR', Gene&Development, Vol. 18(16) pp. 1926-1945, 15 August 2004. See page 1926-1928, 1931	16-21
A	T.A. Hornberger et al., 'The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle', PNAS Vol. 103(12) pp. 4741-4746, 21 March 2006. See abstract, page 4741-4744	16-21

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 NOVEMBER 2007 (13.11.2007)	**13 NOVEMBER 2007 (13.11.2007)**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	PARK, Yeong Gwan
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-8407

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/003754

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/003754

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-15
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-15 pertain to method for treatment of the human body therapy, as well as diagnostic method, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/003754

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO2004026898A1	01.04.2004	KR1020050057260A1	16.06.2005
		US20060172363A1	03.08.2006