Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 16, Issue of April 16, pp. 15719–15722, 2004
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Printed in U.S.A.

Thr^{2446} Is a Novel Mammalian Target of Rapamycin (mTOR) Phosphorylation Site Regulated by Nutrient Status*

Received for publication, December 8, 2003,
and in revised form, February 11, 2004
Published, JBC Papers in Press, February 17, 2004,
DOI 10.1074/jbc.C300534200

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The mammalian target of rapamycin (mTOR) is a key regulator of protein translation. Signaling via mTOR is increased by growth factors but decreased during nutrient deprivation. Previous studies have identified Ser^{2448} as a nutrient-regulated phosphorylation site located in the mTOR catalytic domain, insulin stimulates Ser^{2448} phosphorylation via protein kinase B (PKB), while Ser^{2448} phosphorylation is attenuated with amino acid starvation. Here we have identified Thr^{2446} as a novel nutrient-regulated phosphorylation site on mTOR. Thr^{2446} becomes phosphorylated when CHO-IR cells are nutrient-deprived, but phosphorylation is reduced by insulin stimulation. Nutrient deprivation activates AMP-activated protein kinase (AMPK). To test whether this could be involved in regulating phoshorylation of mTOR, we treated cultured murine myotubes with 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or dinitrophenol (DNP). Both treatments activated AMPK and also caused a concomitant increase in phosphorylation of Thr^{2446} and a parallel decrease in insulin's ability to phosphorylate p70 S6 kinase. *In vitro* kinase assays using peptides based on the sequence in amino acids 2440–2551 of mTOR found that PKB and AMPK are capable of phosphorylating sites in this region. However, phosphorylation by PKB is restricted when Thr^{2446} is mutated to an acidic residue mimicking phosphorylation. Conversely, AMP-kinase-induced phosphorylation is reduced when Ser^{2448} is phosphorylated. These data suggest differential phosphorylation Thr^{2446} and Ser^{2448} could act as a switch mechanism to integrate signals from nutrient status and growth factors to control the regulation of protein translation.

The rate of protein translation is dynamically regulated, being up-regulated at times of cellular growth and attenuated when cells lack nutrients or amino acids (1). Rapamycin attenuates the rate of protein translation and the identification of its cellular target, the mammalian target of rapamycin (mTOR),[1] has greatly increased our understanding of pathways controlling protein translation (2). Activated mTOR stimulates translation by inducing phosphorylation of the eIF4E binding protein (4EBP1) (3–9) and stimulating phosphorylation and activation of p70 S6 kinase (4, 9–11). Nutrient and amino acid deprivation inactivate p70 S6 kinase and promote the dephosphorylation of 4EBP1 via mechanisms that are not fully understood (1, 12–19). mTOR was first identified in yeast (20), and subsequently a single isoform has been cloned in mammals (21–23). The mammalian isoform is a 240-kDa protein, which contains a kinase domain bearing a striking homology to the PI 3-kinase family of lipid kinases. However, mTOR functions only as a protein kinase (24–26), and evidence has been presented that mTOR can directly phosphorylate 4EBP1 and p70 S6 kinase (24, 27, 28). However, the target sequence in both these molecules is very different giving rise to suggestions that the real mechanism for mTOR-mediated changes in phosphorylation may be via an inhibition of protein phosphatase 2A (PP2A) (29).

Rapamycin complexes with the cytosolic receptor FK506-binding protein (FKBP-12), and this complex binds to a distinct region of mTOR upstream of the catalytic domain (22, 30). The finding that a specific monoclonal antibody binding to the extreme C terminus region of mTOR (27) and that deletion of aa 2430–2450 (31) both increased the basal protein kinase activity of mTOR suggested this C-terminal region is of regulatory importance. Evidence has been presented that kinases, including PKB, can phosphorylate this region of mTOR and that such phosphorylation is likely to have a regulatory role (32–34). We and others (17, 31, 35, 36) subsequently identified Ser^{2448} as the PKB-mediated phosphorylation site. The phosphorylation of Ser^{2448} on mTOR is directly related to amino acid and nutrient status (17, 35). More recently it has been suggested that this nutrient effect on mTOR might act through AMPK as activation of this kinase is associated with reduced signaling through mTOR and reduced phosphorylation of Ser^{2448} (36, 37). This suggests that phosphorylation of Ser^{2448} might act as a switch, controlling the activity and function of mTOR and that AMPK may play a role in this process.

We hypothesized this regulation might involve other phosphorylation events in the vicinity of Ser^{2448}. The region surrounding Ser^{2448} is highly conserved in all mammalian sequences for mTOR and contains a number of ser and thr residues that might be regulatory phosphorylation sites as determined by Scansite (38). Here we identified Thr^{2446} as a novel phosphorylation site in mTOR, and we showed that phosphorylation is activated by amino acid deprivation or activation of AMPK while it is attenuated by growth factor stimulation.

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§ Supported by a postgraduate studentship funded by Diabetes United Kingdom.

‖ Supported by the United Kingdom Medical Research Council.

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[1] The abbreviations used are: mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; PI, phosphatidylinositol; aa, amino acid(s); PKB, protein kinase B; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; CHO-IR, Chinese hamster ovary cells stably expressing the human insulin receptor; PBS, phosphate-buffered saline; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; MOPS, 4-morpholinepropanesulfonic acid; AICAR, 5′-aminoimidazole-4-carboxamide ribonucleoside; DNP, dinitrophenol.

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Further evidence is provided that phosphorylation at Thr^{2446} and Ser^{2448} are mutually exclusive indicating these sites may act as switches that integrate the counteracting signals of growth factors and nutrient deprivation.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Phospho-AMPK, total AMPK, and phospho-p70 S6 kinase antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Total p70 S6 kinase and PKC-ζ antibodies and protein G-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK and total ERK was purchased from New England Biolabs (Beverly, MA). PKB antibodies were kindly provided by Dr Dario Alessi, University of Dundee. Rapamycin, PD98059, and okadaic acid were obtained from Calbiochem. AMPK was purified from liver as described previously (39). All other reagents were obtained from Sigma unless stated otherwise. Peptides were generated by the Wolfson Insitute for Biomedical Research (University College London) and were based on the sequence of aa 2440–2551 in mTOR with addition of an N-terminal lysine to improve binding to P81 paper. The peptides used are described in Fig. 5.

mTOR Antibodies—The sheep phospho-mTOR Ser^{2448} antibody (phospho-Ser^{2448}) was as described previously (35). Polyclonal total mTOR antibody was produced in rabbit as described in Withers *et al.* (40) using a glutathione *S*-transferase fusion protein corresponding to the region between amino acids 668 and 939 of the mTOR sequence. Phospho-mTOR Thr^{2446} antiisera was raised in rabbit using the phosphopeptide CSRTRT(P)DSYS, corresponding to amino acids Ser^{2442}–Ser^{2450} of the human mTOR sequence with an N-terminal cysteine added. The antibody was affinity-purified through SulfoLink resin (Pierce) columns. First the antibody was run through a column coupled to the dephosphopeptide, and the unbound was then run-through a second column coupled to the phosphopeptide, and phosphospecific antibodies were eluted.

Cell Culture—Chinese hamster ovary cells stably expressing the human insulin receptor (CHO-IR) were grown in nutrient medium F-12 (Ham), supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution (100×: 10,000 units/ml penicillin, 10 mg/ml streptomycin sulfate, 25 μg/ml amphotericin B). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2. Mouse myoblasts derived from the immortomouse (labeled H2K) were cultured as described previously (39). Insulin stimulation and nutrient deprivation conditions were as described previously (35).

Cell Lysis and Immunoblotting—Cell monolayers were washed once with ice cold Dulbecco's-PBS and lysed at 4 °C in lysis buffer (50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM EDTA, 6 mM EGTA, 1% (v/v) Nonidet P-40, 1 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM Na_3VO_4, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin). Lysates were then centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatents were combined with the appropriate amount of 4 × SDS sample buffer (0.5 M Tris/HCl (pH 6.8), 20% (v/v) glycerol, 8% (w/v) SDS, 2 mM EDTA, 4% (v/v) β-mercaptoethanol, bromphenol blue color). Samples were subject to SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membrane by wet transfer (20 V overnight). The membranes were blotted with the appropriate phospho-antibody overnight at 4 °C in Tris-buffered saline (50 mM Tris, 0.138 M NaCl, 2.7 mM KCl (pH 7.6)) containing 0.1% (v/v) Tween 20 and 1% (w/v) bovine serum albumin. Membranes were blotted for total antibody for 1 h in TBS containing 0.1% (v/v) Tween 20 and 1% (w/v) dried milk powder. Following incubation with the primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence in accordance with the manufacturer's instructions (Amersham Biosciences), and visualized with a luminescent image analyzer; images were captured using a cooled CCD camera system (Fuji LAS1000)

Peptide Assay—Confluent CHO-IR cells were serum-starved overnight with Ham's F-12 medium containing 1% antibiotic-antimycotic solution. Cells were stimulated with 100 nM insulin for 10 min then lysed in PKB lysis buffer (50 mM HEPES, 0.2 mM EDTA, 2.2 mM EGTA, 100 mM KCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 nM okadaic acid). Following centrifugation as described above, supernatents were pooled then equal volumes were immunoprecipitated with anti-C-terminal PKB for 2 h. Immunocomplexes were then incubated with protein G-agarose for 45 min. Immunoprecipitates were washed and assayed as described previously (41). Kinases were incubated with 1 mM peptide, 1 μM protein kinase inhibitor, 20 mM $MgCl_2$, 100 μM cold ATP:5 μCi/reaction [γ-^{32}P]ATP in assay buffer (20 mM MOPS (pH 7.0), 1 mM EDTA, 1 mM EGTA, 0.01% Triton X-100, 5% (v/v) glycerol) in a final reaction volume of 30 μl. AMPK samples also contained 1 mM AMP. Reactions were incubated at 30 °C for 15 min with agitation then stopped by spotting all the reaction mixture onto P81 grade phosphocellulose paper (Whatman) and washed five times in 75 mM *ortho*-phosphoric acid. Papers were dried and transferred into scintillation vials for liquid scintillation counting (Beckman).

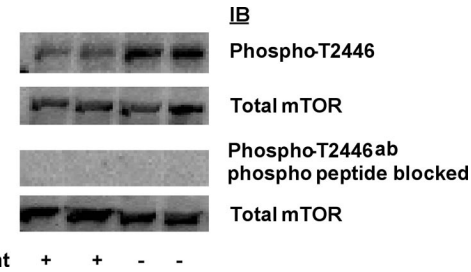

FIG. 1. **Phosphorylation of mTOR on Thr^{2446} increases with nutrient deprivation.** CHO-IR cells were grown in Ham's F-12 medium containing 10% fetal calf serum and where indicated were starved in Dulbecco's-PBS for 60 min. Cell lysates were separated by SDS-PAGE and Western blotted as indicated with either affinity-purified mTOR phospho-Thr^{2446} antibody, the phospho-Thr^{2446}-mTOR antibody blocked with phosphopeptide, or an antisera that recognized total levels of mTOR.

RESULTS

Inverse Relationship between Phosphorylation of Thr^{2446} and Ser^{2448} on mTOR—Here we have developed an affinity-purified antibody that recognizes phospho-Ser^{2446} in mTOR. The antibody identifies a band that corresponds to the molecular weight recognized by antibodies that recognize total mTOR, and the reactivity of this band with the phospho-antibody band is blocked by co-incubation with the phosphopeptide that was used to make the antibody (Fig. 1), while the dephosphopeptide has no effect on immunoreactivity of the phospho-antibody (data not shown). While total levels of mTOR did not change with nutrient deprivation, we find that immunoreactivity with the Thr^{2446} phospho-antibody increases when cells are deprived of nutrient (Fig. 1). Furthermore we find that the rapid increase in reactivity to the phospho-Thr^{2446} antibody during nutrient deprivation inversely parallels the decrease in reactivity to the phospho-Ser^{2448} antibody under the same starvation conditions (Fig. 2*A*). The reverse was observed when CHO-IR cells were stimulated with insulin with a decrease in reactivity with the phospho-Thr^{2446} antibody observed with prolonged stimulation with insulin corresponding to an increase in reactivity to the phospho-Ser^{2448} antibody under the same conditions (Fig. 2*B*). Insulin-mediated activation of Ser^{2448} phosphorylation requires activation of PI 3-kinase and PKB (35). Here we find that treatment with the PI 3-kinase inhibitor wortmannin, which blocks the activation of PKB, blocks the serum-induced phosphorylation on Ser^{2448} but has no effect on nutrient induced increases in Thr^{2446} phosphorylation (Fig. 3).

Effect of AMPK Activators on mTOR Phosphorylation—AMPK is activated when ATP/AMP ratios fall (42), and the phosphorylation of AMPK was increased following aa withdrawal in CHO-IR cells (data not shown). Therefore to determine the physiological significance of nutrient status in regulating mTOR Thr^{2446} phosphorylation, mouse H2K muscle cells were treated with dinitrophenol, a metabolic poison that rapidly decreases cellular ATP level, or with AICAR riboside, a cell-permeable compound that in some cell types, including the H2Ks, is degraded to ZMP, which in turn is capable of activating AMPK. Activity (Fig. 4) of AMPK were increased by both AICAR and DNP in these cells. Both treatments also cause an acute increase in Thr^{2446} phosphorylation, even in the presence

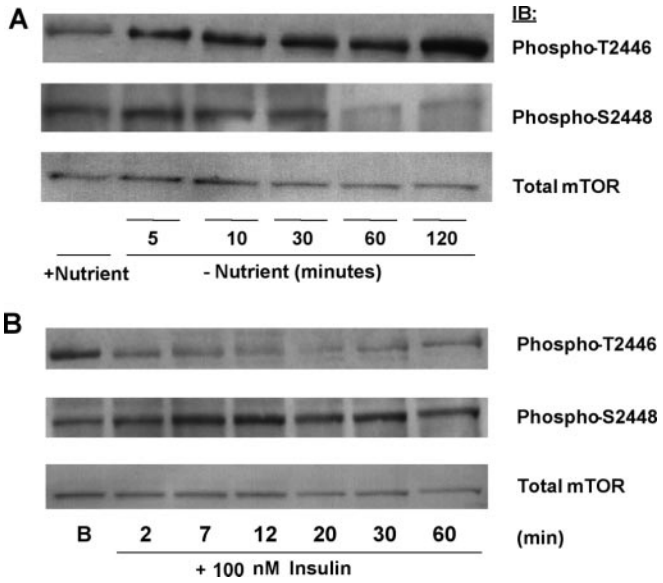

FIG. 2. **Inverse phosphorylation of mTOR Thr^{2446} and Ser^{2448} with nutrient deprivation and insulin stimulation.** *A*, CHO-IR cells were grown in Ham's F-12 containing 10% FCS and then deprived of nutrients in Dulbecco's-PBS for the indicated time. *B*, CHO-IR cells were serum-starved overnight in Ham's F-12 medium and then exposed to 100 nM insulin for the indicated time. In all cases cell lysates were separated by SDS-PAGE, transferred to PVDF, and then Western blotted with the antibodies as shown. Results are shown in duplicate, and similar results were obtained in three independent experiments.

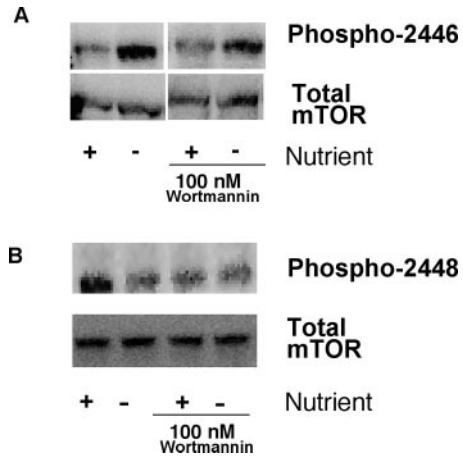

FIG. 3. **Effects of wortmannin treatment on mTOR Thr^{2446} and Ser^{2448} phosphorylation in CHO-IR cells.** CHO-IR cells were fed with Ham's F-12 supplemented with 10% fetal calf serum or nutrient-starved in PBS for 1 h where indicated in the presence of absence of 100 nM wortmannin. In all cases cells were lysed, and lysates were separated by SDS-PAGE, wet-transferred to PVDF, and then blotted with the antibodies as shown. Similar results were obtained in three independent experiments.

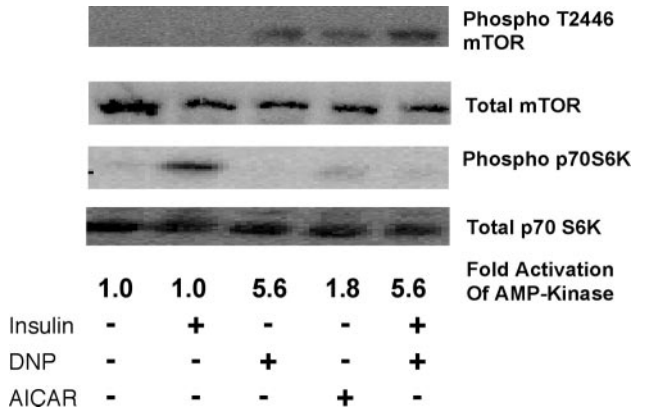

FIG. 4. **Effects of AMPK activators on Thr^{2446} phosphorylation in H2K myoblasts.** Differentiated H2K myotubes were starved overnight in serum-free medium. Cells were stimulated with 100 nM insulin, 0.5 mM DNP, 0.5 mM AICAR for 30 min as indicated. In all cases cells were lysed, and 1 aliquot of lysate was assayed for AMPK activity, and the fold stimulation over basal is shown. Another aliquot of lysate containing 100 μg of protein was separated by SDS-PAGE, wet-transferred to PVDF, and then blotted with the antibodies as shown. Similar results were obtained in three independent experiments.

Peptide	Sequence
Wild Type	KKRSRTRTDSYSA
T2446E	KKRSRTREDSYSA
T2446A	KKRSRTRADSYSA
Phospho-S2448	KKRSRTRTDS(P)YSA

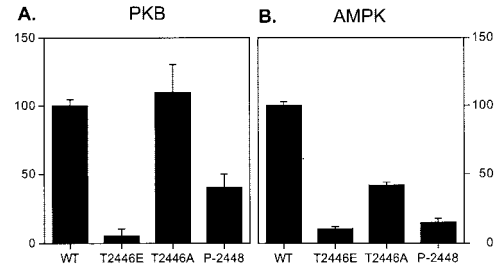

FIG. 5. **Phosphorylation of mTOR-derived peptides by PKB and AMPK *in vitro*.** Immunoprecipitates of PKB (*A*) or recombinant AMPK (*B*) were assayed against all the peptide sequences shown above as indicated under "Experimental Procedures." The phosphorylation of each peptide was compared with the phosphorylation of the wild type peptide by the indicated kinase. Results shown are $n = 3$ in triplicate $\pm$ S.E.

of insulin. This is matched by an attenuation of insulin stimulation of p70 S6 kinase phosphorylation (Fig. 4).

Phosphorylation of mTOR Regulatory Region—In an effort to identify kinases capable of phosphorylating this region, an *in vitro* kinase assay approach was taken using a range of peptides based on aa 2440–2551 of mTOR (Fig. 5). p70 S6 kinase phosphorylated all the peptides with efficiency greater than or equal to the phosphorylation of the wild type peptide (data not shown) suggesting that this kinase was phosphorylating various sites in the peptide and that its action did not depend on the status of the Thr^{2446} site. However, phosphorylation of the T2446E peptide by PKB and AMPK was very low compared with phosphorylation of wild type peptides. PKB phosphorylated the T2446A peptide equally as well as it phosphorylated wild type, while the phospho-Ser^{2448} was phosphorylated at ~20% of wild type level. AMP kinase-mediated phosphorylation of the T2446E and T2446A peptide was greatly reduced compared with the wild type peptide consistent with Thr^{2446} being a major AMPK target site, while phosphorylation at the 2448 position virtually abolished the ability of AMPK to phosphorylate the peptides (Fig. 5).

DISCUSSION

The results presented here provide evidence that Thr^{2446} is a regulated phosphorylation site in mTOR, and given the previous evidence that this is an important regulatory region (27, 31, 43), this phosphorylation is likely to play a regulatory role in mTOR action. The Thr^{2446} phosphorylation is likely to be a negative regulation given that Thr^{2446} phosphorylation is associated with conditions that reduce signaling through mTOR and our finding that phosphorylation of Thr^{2446} correlates with an attenuation of insulin-stimulated phosphorylation of p70 S6 kinase.

We have previously provided evidence that Ser^{2448} is phosphorylated downstream of PKB (35). Despite the fact Thr^{2446} fits a weak PKB consensus sequence, our evidence indicates

PKB is not the kinase normally phosphorylating Thr^{2446} as the T2446A peptide is phosphorylated well by PKB and insulin, which activates PKB, reduces Thr^{2446} phosphorylation. Furthermore, wortmannin, which blocks activation of PKB and blocks Ser^{2448} phosphorylation, does not reduce phosphorylation at Thr^{2446}. Recent evidence has linked AMPK to regulation of mTOR (36, 37), and our results suggest that AMPK is likely to be at least one of the kinases regulating phosphorylation at Thr^{2446}. There are two lines of evidence for this; first, nutrient deprivation, DNP, and AICAR all activate AMPK and stimulate phosphorylation of Thr^{2446}, and second, AMPK can directly phosphorylate the aa 2440–2551 mTOR peptide. The optimum amino acid sequence for AMPK has not been fully defined, but our peptide phosphorylation studies provide evidence that Thr^{2446} is likely to be phosphorylated by the enzyme; while our peptides contain three serines and two threonines the mutation of Thr^{2446} residue to glutamic acid or alanine greatly reduced phosphorylation of the peptide by AMPK.

Our data also show that conditions that increase phosphorylation at Thr^{2446} tend to decrease phosphoprylation at Ser^{2448} and vice versa. This could be in part due to coordinate regulation of the kinases involved. For example insulin will activate PKB while reducing AMPK activity. However, our data suggest a further level of regulation as it suggests that phosphorylation of the two sites is mutually exclusive. This is supported by the finding that PKB does not phosphorylate the aa 2440–2551 peptide when Thr^{2446} is changed to glutamic acid (mimicking phosphorylation), but phosphorylation is normal when Thr^{2446} is changed to alanine. Furthermore, while Ser^{2448} is clearly not a major AMPK-stimulated phosphorylation site, phosphorylation of the peptide by AMPK is greatly reduced by phosphorylation at the 2448 position, consistent with the notion that phosphorylation at Thr^{2446} and Ser^{2448} are mutually exclusive events.

The mutual exclusivity probably arises due to the phosphorylation effecting the substrate recognition sequence for the second kinase, and interestingly, there is a precedent for mutually exclusive regulatory phosphorylation sites separated by a single amino acid being involved in integrating signals related to nutrient status in other enzymes. In acetyl-CoA carboxylase this involves Ser^{77}, which is an AMPK site, Ser^{79}, which is a proetin kinase A target (44), and in the case of hormone-sensitive lipase, it involves Ser^{563} as a protein kinase A site and Ser^{565} as an AMPK site (45, 46).

In summary, we identify Thr^{2446} as a novel phosphorylation site in mTOR that is increased by conditions of low nutrient and correlates with negative regulation of mTOR activity. As phosphorylation at this site limits phosphorylation at Ser^{2448} and vice versa, we suggest that these two phosphorylation sites might act as a switch to control the positive and negative signals regulating protein translation.

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