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 phosphorylation 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 of 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, 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 threonine 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.

\(^{1}\)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.
Further evidence is provided that phosphorylation at Thr\(^{2446}\) and Ser\(^{2446}\) 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-\(\zeta\) antibodies and protein G-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK and total ERK were purchased from New England Biolabs (Beverly, MA). PKB antibodies were kindly provided by Dr. Dario Alessi, University of Dundee. Rapamycin, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and streptomyccin sulfate were obtained from Sigma unless stated otherwise. Peptides were generated by the Wolfson Institute for Biomedical Research (University College London) and were based on the sequence of an N-terminal lysine to improve binding to PS1 paper. The peptides used are described in Fig. 5.

**mTOR Antibodies—**The sheep phospho-mTOR Ser\(^{2446}\) antibody (phospho-Ser\(^{2446}\)) 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\(^{2446}\) antisera was raised in rabbit using the phosphopeptide CSRTRT(P)DSYS, corresponding to amino acids Ser\(^{2442}\)–Ser\(^{2446}\) 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 phosphopeptide 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 \times 10,000 units/ml penicillin, 10 mg/ml streptomycin sulfate, 25 mg/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 (35). 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\(_3\)VO\(_4\), 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin). Lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatants were combined with the appropriate amount of 4 × SDS sample buffer (0.5 mM 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 mM NaCl, 27 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 in Ham’s F-12 medium containing 1% antibiotic-antimycotic solution. Cells were stimulated with 100 mM 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 mg/ml ascorbic acid). Following centrifugation as described above, supernatants were pooled then equal volume aliquots were precipitated with anti-C-terminal PKB (PDK2) antibody identified a band that corresponds to the molecular weight of mTOR. 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\(^{2446}\) 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\(^{2446}\) antibody under the same starvation conditions (Fig. 2A). 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\(^{2446}\) antibody under the same conditions (Fig. 2B).

**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 of 15720-
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 Thr2446 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-Ser2448 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 Thr2446 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 Thr2446 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 Thr2446 phosphorylation is likely to be a negative regulation given that Thr2446 phosphorylation is associated with conditions that reduce signaling through mTOR and our finding that phosphorylation of Thr2446 correlates with an attenuation of insulin-stimulated phosphorylation of p70 S6 kinase.

We have previously provided evidence that Ser2448 is phosphorylated downstream of PKB (35). Despite the fact Thr2446 fits a weak PKB consensus sequence, our evidence indicates...
PKB is not the kinase normally phosphorylating Thr\textsuperscript{2446} as the T2446A peptide is phosphorylated well by PKB and insulin, which activates PKB, reduces Thr\textsuperscript{2446} phosphorylation. Furthermore, wormtannin, which blocks activation of PKB and blocks Ser\textsuperscript{2448} phosphorylation, does not reduce phosphorylation at Thr\textsuperscript{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\textsuperscript{2446}. There are two lines of evidence for this: first, nutrient deprivation, NPD, and AICAR all activate AMPK and stimulate phosphorylation of Thr\textsuperscript{2446}, and second, AMPK can directly dephosphorylate 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\textsuperscript{2446} is likely to be phosphorylated by the enzyme; while our peptides contain three serines and two threonines the mutation of Thr\textsuperscript{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\textsuperscript{2446} tend to decrease phosphorylation at Ser\textsuperscript{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\textsuperscript{2446} is changed to glutamic acid (mimicking phosphorylation), but phosphorylation is normal when Thr\textsuperscript{2446} is changed to alanine. Furthermore, while Ser\textsuperscript{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\textsuperscript{2446} and Ser\textsuperscript{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\textsuperscript{565} as an AMPK site and Ser\textsuperscript{565} as an AMPK site (45, 46).

In summary, we identify Thr\textsuperscript{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\textsuperscript{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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