Control of Ser\textsuperscript{2448} Phosphorylation in the Mammalian Target of Rapamycin by Insulin and Skeletal Muscle Load*

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We have investigated the effects of insulin, amino acids, and the degree of muscle loading on the phosphorylation of Ser\textsuperscript{2448} in a site in the mammalian target of rapamycin (mTOR) phosphorylated by protein kinase B (PKB) \textit{in vitro}. Phosphorylation was assessed by immuno blotting with a phosphospecific antibody (anti-Ser\textsuperscript{P2448}) and with mTAb1, an activating antibody whose binding is inhibited by phosphorylation in the region of mTOR that contains Ser\textsuperscript{2448}. Incubating rat diaphragm muscles with insulin increased Ser\textsuperscript{2448} phosphorylation but did not change the total amount of mTOR. Insulin, but not amino acids, activated PKB, as evidenced by increased phosphorylation of both Ser\textsuperscript{308} and Thr\textsuperscript{472} in the kinase. Ser\textsuperscript{2448} phosphorylation was also modulated by muscle-loading. Overloading the rat plantaris muscle by synergist muscle ablation, which promotes hypertrophy of the plantaris muscle, increased Ser\textsuperscript{2448} phosphorylation. In contrast, unloading the gastrocnemius muscle by hindlimb suspension, which promotes atrophy of the muscle, decreased Ser\textsuperscript{2448} phosphorylation, an effect that was fully reversible. Neither overloading nor hindlimb suspension significantly changed the total amount of mTOR. In summary, our results demonstrate that atrophy and hypertrophy of skeletal muscle are associated with decreases and increases in Ser\textsuperscript{2448} phosphorylation, suggesting that modulation of this site may have an important role in the control of protein synthesis.

Skeletal muscle mass is controlled by several factors including insulin, amino acids, and the degree of muscular activity (1–4). Muscle wasting is a hallmark of untreated Type 1 diabetes mellitus in humans (5), as well as in experimentally induced diabetes in animals (6). Restoring insulin to diabetics blocks the loss of muscle protein in humans (5) and increases muscle protein synthesis in animal models of diabetes (7–9). Muscle wasting is a hallmark of untreated Type 1 diabetes mellitus in humans (5), as well as in experimentally induced diabetes in animals (6). Restoring insulin to diabetics blocks the loss of muscle protein in humans (5) and increases muscle protein synthesis in animal models of diabetes (7–9). Although a net accumulation of protein cannot occur without an adequate supply of the precursor amino acids, certain amino acids exert a control on the rate of protein synthesis through a more specific mechanism. Branched chain amino acids, leucine in particular, stimulate protein synthesis by activating a nutrient-sensing pathway (4). Increasing the load on a muscle promotes hypertrophy (10), and unloading causes atrophy (11).

The effects of insulin, amino acids, and loading involve changes in the rates of muscle protein synthesis (4, 12, 13), a process that involves mRNA translation. The stimulatory effects on mRNA translation are mediated in part by the phosphorylation of the mRNA translational regulators, PHAS-I and p70\textsuperscript{S6K} (4). Nonphosphorylated PHAS-I binds to eIF4E,\textsuperscript{1} the mRNA cap-binding protein, and prevents eIF4E from interacting with eIF4G. When phosphorylated in the appropriate sites, PHAS-I dissociates from eIF4E, allowing eIF4E to bind eIF4G to form an initiation complex needed for efficient binding and/or scanning by the 40 S ribosomal subunit. p70\textsuperscript{S6K} is a mitogen-activated protein kinase whose activation has been proposed to promote increased translation of messages, which have a polypyrimidine motif just downstream of the 5′ cap (4).

Both PHAS-I and p70\textsuperscript{S6K} are controlled by mTOR, the 245-amino acid mammalian counterpart of the tor1p and tor2p proteins that control protein synthesis and cellular growth in \textit{Saccharomyces cerevisiae} (14). mTOR functions as a growth factor and nutrient-sensing signaling molecule in mammalian cells. Defining the role of mTOR has been greatly facilitated by a highly specific inhibitor, rapamycin. To inhibit mTOR rapamycin requires as a partner the FK506-binding protein, FKBP12, because it is only when complexed with FKBP12 that rapamycin is able to bind mTOR with high affinity (15). This high affinity interaction has been exploited to purify mTOR from mammalian tissues and cell lines (see for examples Refs. 16 and 17). The effects of both insulin and amino acids on increasing the phosphorylation of PHAS-I and p70\textsuperscript{S6K} in skeletal muscle are attenuated by rapamycin (18). Recently, rapamycin was shown to block both the compensatory hypertrophy of the plantaris muscle resulting from synergist ablation \textit{in vivo} and the increase in muscle mass associated with recovery from disuse atrophy (2). These findings with rapamycin have implicated mTOR in the control of skeletal muscle mass by muscle loading.

The mechanisms through which mTOR signals and how mTOR activity is controlled are unclear. mTOR is able to phosphorylate both PHAS-I and p70\textsuperscript{S6K} \textit{in vitro} (16, 19), although it is still not certain whether mTOR phosphorylates these proteins in cells. There is evidence that mTOR is controlled by PKB, which is activated in response to phospholipid products of the phosphatidylinositol 3-kinase reaction. PKB is able to phosphorylate Ser\textsuperscript{2448} in mTOR \textit{in vitro} (20, 21), and this site becomes phosphorylated in response to PKB activation in cells (20–22). Phosphorylation of this region of mTOR was first detected using the antibody, mTAb1, whose reactivity with mTOR was decreased in response to phosphorylation (22).

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** The abbreviations used are: eIF, eukaryotic initiation factor; mTOR, mammalian target of rapamycin; GST, glutathione S-transferase; HEK, human embryonic kidney cells; PKB, protein kinase B.
sequently, phosphorylation of Ser\textsuperscript{2448} was confirmed by using phosphospecific antibodies to this site (20, 21).

Interestingly, there is other evidence that the region surrounding Ser\textsuperscript{2448} is an important regulatory domain in mTOR. Binding of the antibody, mTAb1, to this region markedly increases the protein kinase activity of mTOR \textit{in vitro} (21, 22). Also, recombinant mTOR in which the region containing the mTAb1 epitope has been deleted exhibits increased activity, both \textit{in vivo} and \textit{in vitro} (21). These findings suggest that phosphorylation of Ser\textsuperscript{2448} may be important in controlling mTOR. Therefore, we conducted experiments to determine whether treatments known to modulate skeletal muscle mass affect the phosphorylation of Ser\textsuperscript{2448}.

**EXPERIMENTAL PROCEDURES**

**Isolation and Incubation of Rat Diaphragm Muscles**—All incubations were performed at 37 °C in medium continuously bubbled with a 19:1 mixture of O\textsubscript{2}/CO\textsubscript{2}, essentially as described previously (18). Briefly, male Sprague-Dawley rats (∼150 g) were sacrificed by decapitation, and diaphragm muscles were excised and incubated in Dulbecco’s modified Eagle medium (30 ml/muscle) for 45 min to remove endogenous hormones. The muscles were then incubated with or without insulin (250 μM) and amino acids in Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 1.2 mM potassium phosphate, and 25 mM NaHCO\textsubscript{3}, pH 7.4) containing 5 mM glucose. After 20 min the muscles were blotted on filter paper and immediately frozen in liquid N\textsubscript{2}.

**Skeletal Muscle Loading and Unloading**—The procedures for loading plantaris muscles by synergist ablation and unloading gastrocnemius muscles by hindlimb suspension were conducted exactly as described previously (2). Briefly, rats (Sprague-Dawley, ∼250 g) were anesthetized with ketamine/xylazine (50:10 mg/kg, intraperitoneal). For synergist ablation, the soleus, medial gastrocnemius, and lateral gastrocnemius muscles were removed bilaterally using aseptic surgical techniques (2). This produces a functional overload on the plantaris muscles (10). For hindlimb suspension studies, anesthetized animals were fitted with a tail-traction bandage. Following recovery from the anesthetic, a swivel hook was placed through the bandage and raised so that the hindlimbs were suspended just off the cage floor (11). At the appropriate time after surgery, the rats were sacrificed, and the plantaris muscles or gastrocnemius muscles were removed, frozen in liquid N\textsubscript{2}, and stored at -80 °C. Muscles from weight-matched surgically untreated rats served as controls.

**Affinity Purification of mTOR**—Crude extracts of skeletal muscle contain large amounts of myosin heavy chain, which comigrates with mTOR and prevents detection of mTOR by immunoblotting. Therefore, mTOR was affinity-purified prior to electrophoresis. The frozen muscles were manually ground with a porcelain mortar and pestle chilled in liquid N\textsubscript{2}. Powdered tissue was homogenized on ice using a motor-driven tissue grinder (Teflon-glass) in 3 ml of homogenization buffer, which was composed of Buffer A (50 mM NaCl, 10 mM NaF, 0.25% Tween 20, 10% glycerol, 0.1 mM dithiothreitol, 500 mM microcystin-LR, 50 mM Tris/HCl, pH 7.4) supplemented with 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, leupeptin, and pepstatin-A. The homogenates were rotated at 4 °C for 1 h and then centrifuged at 8,900 × g for 30 min at 4 °C. The protein concentrations of the supernatants were determined by the BCA method (Pierce).

To purify mTOR, 100 μg of a recombinant glutathione S-transferase (GST) FKBP12 fusion protein (GST-FKBP12), prepared as previously described (16, 17), were incubated with 25 μl of glutathione-Sepharose beads in Buffer B (145 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 10% bovine serum albumin for 1 h at 21 °C. The beads were then washed three times with homogenization buffer (1 ml/wash) and incubated with 1 ml of muscle extract (1 mg of protein) plus 10 μM rapamycin (Calbiochem) or where indicated, 10 μM FK506 (Fujisawa Pharmaceuticals). After 90 min at 4 °C, the beads were washed twice with Buffer A, twice with Buffer A plus 500 mM NaCl, and then twice in Buffer A.

**Antibodies**—The mTOR antibodies mTAb1 and mTAb2 are the same as used in previous studies (16) and were generated by immunizing rabbits with peptides having sequences corresponding to residues 2433–2450 and 1272–1290, respectively, in mTOR. Antibodies to the β-isof orm of PKB were from Upstate Biotechnology. Phosphospecific antibodies to the Ser\textsuperscript{2448} and Thr\textsuperscript{2451} sites in PKB were from New England Biolabs.

To generate phosphospecific antibody to the Ser\textsuperscript{2448} site in mTOR, essentially the same procedures were used as described previously for the preparation of phosphospecific antibodies to sites in PHAS-I (23) except that different peptides were used. Briefly, rabbits were immunized with a phosphopeptide (CTRTS\textsubscript{2}/YSAGQS, where S\textsubscript{2} is phosphoserine) coupled to keyhole limpet hemocyanin. After the second booster injection, serum was collected and incubated with an affinity resin prepared by coupling a nonphosphorylated peptide to Sulfo-Link beads (Pierce). The unbound fraction was then incubated with a phosphopeptide resin. After exhaustively washing the resin, the anti-Ser(P)\textsuperscript{2448} antibodies were eluted with 0.3 mM glycine/HCl (pH 2.7), neutralized, and further purified with protein A-Sepharose.

**Electrophoretic Analyses and Immunoblotting of mTOR and PKB**—Samples of affinity-purified mTOR were subjected to SDS-PAGE (7.5% polyacrylamide gel) (24). The proteins were then electrophoretically transferred to Immobilon membranes and immunoblotted with mTOR and PKB antibodies as described previously (16, 22). After washing the membranes, the light generated by the alkaline phosphatase-conjugated secondary antibody and Tropix reagent was detected using x-ray film (Kodak XAR-5). Relative signal intensities of the mTOR bands were determined using a scanning laser densitometer (Molecular Dynamics). To assess mTOR phosphorylation relative to total mTOR, immunoblots prepared with anti-Ser(P)\textsuperscript{2448} or mTAb1 were stripped and reprobed with mTAb2, whose binding is not affected by phosphorylation of Ser\textsuperscript{2448}. After correcting for the total amount of mTOR present, the statistical significance of differences among groups was assessed by analysis of variance and the Fisher least significant difference post-hoc test (25). An α level of p < 0.05 was accepted for statistical significance.

**RESULTS**

**Identification of mTOR in Skeletal Muscle**—mTOR was affinity-purified from skeletal muscle extracts by using GST-FKBP12 rapamycin resin (16, 17) before immunoblots were prepared. mTOR appeared as a single band when immunoblotted with mTAb2 (Fig. 1, lane 1). The FKBP12/FK506 complex does not bind to mTOR (15). Therefore, as a control, the affinity purification procedure was conducted in an identical manner except that FK506 was substituted for rapamycin. As expected, no mTOR was detected in the sample generated with FK506 (Fig. 1, lane 2).

**Effects of Insulin and/or Amino Acids on Ser\textsuperscript{2448} Phosphorylation**—Because insulin and amino acids were known to modulate downstream effectors in the mTOR pathway, we conducted experiments to determine whether incubating muscles with these agents affected the phosphorylation of Ser\textsuperscript{2448} in mTOR. We chose to use hemidiaphragms for these experiments because these muscles may be maintained in a viable, insulin-responsive state during short term incubations \textit{in vitro}. Phosphorylation of Ser\textsuperscript{2448} was assessed by immunoblotting with the phosphospecific antibody, anti-Ser(P)\textsuperscript{2448} mTOR that had been affinity-purified from control cells was readily detected with the anti-Ser(P)\textsuperscript{2448} antibody, indicating that this site co-
tained some phosphate even in the basal state (Fig. 2A). Incubating muscles with either a mixture of amino acids or insulin increased Ser<sup>2448</sup> phosphorylation by −1.5- or 2-fold, respectively (Fig. 2B). The combination of insulin plus amino acids was no more effective in increasing Ser<sup>2448</sup> phosphorylation than insulin alone.

Immunoblots were also prepared with mTAb1, whose binding is decreased by phosphorylation of one or more sites in the regulatory region surrounding Ser<sup>2448</sup>. Insulin plus amino acids decreased the mTAb1 signal by −40% (Fig. 2C). Although the results were somewhat more variable than that observed with the anti-Ser(P)<sup>2448</sup> antibody, the findings with mTAb1 provide independent confirmation that the phosphorylation of mTOR was increased by insulin. To determine whether the treatments affected the total amount of mTOR present, the blots were stripped and reprobed with mTAb2, whose binding is not affected by phosphorylation of Ser<sup>2448</sup>. The reactivity of mTOR with mTAb2 was not significantly changed by insulin or amino acids (Fig. 2D).

Because PKB has been shown to phosphorylate mTOR on Ser<sup>2448</sup> in vitro (20, 21), we investigated the abilities of insulin and amino acids to activate this kinase. This was accomplished by using phosphospecific antibodies to assess changes in the phosphorylation of Ser<sup>308</sup> and Thr<sup>473</sup>. These two sites become phosphorylated when PKB binds phospholipid products of the phosphatidylinositol 3-kinase reaction, and phosphorylation of both is required for full activation of the kinase (26). Insulin markedly increased the phosphorylation of both Ser<sup>308</sup> and Thr<sup>473</sup> (Fig. 3). In contrast, amino acids, either alone or in combination with insulin, had little if any effect on PKB phosphorylation (Fig. 3).

**Effects of Skeletal Muscle Loading on Ser<sup>2448</sup> Phosphorylation**—Recent findings have implicated mTOR in the control of muscle fiber size in response to changes in load (2). Therefore, we conducted experiments to investigate the effect of loading on the phosphorylation of mTOR. In the first series of experiments, we investigated the phosphorylation of Ser<sup>2448</sup> in a synergist ablation model of compensatory hypertrophy. In this model, the gastrocnemius and soleus muscles are surgically removed. This results in overloading of the plantaris muscle, which undergoes compensatory hypertrophy (10, 13). Under the conditions of the present experiments, the weight of the plantaris muscle was increased by 40% (2). In Fig. 4A, samples of plantaris muscles from control animals and samples obtained 2 weeks following synergist ablation were immunoblotted with mTOR antibodies. Immunoreactivity with the phosphospecific antibody to Ser(P)<sup>2448</sup> was increased more than 2-fold by overloading the muscle (Fig. 4B). A reciprocal change was observed in the mTAb1 signal, although the effect was somewhat smaller than that observed with the anti-Ser(P)<sup>2448</sup> antibody (Fig. 4C). Immunoblotting with mTAb2 indicated that the extract samples contained approximately equal amounts of mTOR (Fig. 4D). Thus, the changes in anti-Ser(P)<sup>2448</sup> and mTAb1 binding indicate that synergist ablation increased the phosphorylation of mTOR.

Because increasing muscle load increased phosphorylation of mTOR, we were interested in determining whether decreasing load would decrease phosphorylation of the protein. To investigate this possibility, rats were suspended by their tails to unload their hindlimbs. Fourteen days of this treatment decreased the weight of the gastrocnemius muscle by 35% (2). Unloading the gastrocnemius muscles in this manner decreased the phosphorylation of Ser<sup>2448</sup> by −60% (Fig. 5, A and B).

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2 Although the amino acid and control values were judged to be not significantly different by an analysis of variance test, p was less than 0.05 by Student’s t test.
B). Restoring load on the muscles by removing the suspension apparatus fully reversed the effect of unloading. Hindlimb suspension was without effect on binding of mTAb1 (Fig. 5C) or on the total amount of mTOR (Fig. 5D).

**DISCUSSION**

Insulin and muscle loading were found to increase the phosphorylation of Ser2448. These findings provide the first direct evidence of modification of mTOR in skeletal muscle and have potentially important implications in the control of muscle protein synthesis.

Previous results suggested that Ser2448 is important in the control of mTOR activity. Brunn et al. (16) demonstrated that mTAb1, which binds to the region of mTOR containing this site, markedly increased the PHAS-I kinase activity of mTOR. Scott et al. (22) later noted that insulin promoted the phosphorylation of mTOR in this region and proposed that phosphorylation of Ser2448 activated mTOR (22). Next, Sekulic et al. (21) generated a mutant recombinant mTOR lacking the 20-amino acid mTAb1 epitope and discovered that the mutant enzyme exhibited enhanced protein kinase activity. Based on these observations, it was suggested the region containing the epitope was an inhibitory regulatory domain and that phosphorylation of Ser2448 might activate mTOR by relieving the inhibition. Activation of mTOR would be expected to lead to increased phosphorylation of the downstream effectors, PHAS-I and p70S6K, resulting in increased protein synthesis.

Results from mutagenesis studies have cast some doubt on the significance of Ser2448 phosphorylation. Sekulic et al. (21) investigated the effects of overexpressing an mTOR protein having an Ala2448 mutation on the phosphorylation of p70S6K and PHAS-I in HEK293 cells. The effects of the Ala2448-mutated mTOR in supporting activation of p70S6K in response to insulin were no different from those of mTOR with Ser2448. This finding would argue strongly against a role of Ser2448 phosphorylation as an important regulator of mTOR function were it not for the fact that the overexpression studies were conducted with forms of mTOR rendered rapamycin-resistant by mutating Ser2035 to Ile. We have recently discovered that this mutation markedly inhibits the ability of mTOR to phosphorylate PHAS-I in vitro.3 Thus, preventing phosphorylation of Ser2448 by the Ala2448 mutation in rapamycin-resistant mTOR (Ser2035 to Ile mutation) would not be expected to decrease activity toward PHAS-I, because this form of mTOR already exhibits reduced activity. Additional studies will be needed to address the functional effect of Ser2448 phosphorylation on mTOR activity.

The effects of insulin on increasing Ser2448 in hemidiaphragms were associated with activation of PKB (Fig. 3). This finding is consistent with other studies, which have generated three main lines of evidence supporting the conclusion that the phosphorylation of Ser2448 is controlled by PKB (20, 21). First, Ser2448 and the surrounding sequence is a good fit to the consensus motif (RXRXX(S/T)h), where X is any amino acid and...
h is a hydrophobic residue) for phosphorylation by PKB (27), and Ser$^{2448}$ can be phosphorylated by purified PKB in vitro (20, 21). Second, increasing PKB in cells is associated with increased phosphorylation of Ser$^{2448}$. Insulin activated endogenous PKB activity and increased the phosphorylation of Ser$^{2448}$ in HEK293 cells, CHOIR800 cells, and 3T3L1 adipocytes (20, 22). Moreover, overexpressing a constitutively active PKB in HEK293 cells or activating a PKB-estrogen fusion protein with tamoxifen in MER-Akt cells decreased mTab1 binding, suggestive of increased phosphorylation of Ser$^{2448}$ (22). Third, inhibition of PKB is associated with decreases in Ser$^{2448}$ phosphorylation. In 3T3L1 adipocytes, phosphorylation of Ser$^{2448}$ was blocked by inhibitors of phosphatidylinositol 3-kinase, which also block activation of PKB (22). Also, overexpressing a kinase-dead PKB in HEK293 cells blocked Ser$^{2448}$ phosphorylation (21).

Like insulin, the degree of muscle loading can alter mRNA translation, protein synthesis, and muscle mass (1, 2, 12, 13). Load-dependent changes in skeletal muscle size are partly explained by changes in mRNA translation (1–3). Muscle atrophy induced by hindlimb suspension (2, 3) is associated with a decrease in the phosphorylation of p70$^{S6K}$, whereas, muscle hypertrophy following resistance training is associated with an increase in p70$^{S6K}$ phosphorylation (1). Furthermore, overload-induced hypertrophy of the rat plantaris muscle is associated with an increase in p70$^{S6K}$ activity and a decrease in PHAS-1 bound to eIF4E, indicating an increase in mRNA translation initiation (2). Interestingly, the changes in Ser$^{2448}$ phosphorylation observed with muscle loading and unloading correlate well with the changes in PKB phosphorylation noted by Bodine et al. (2). Thus, both muscle mass and PKB phosphorylation were decreased in gastrocnemius muscles following hindlimb suspension, and synergist ablation resulted in a severalfold increase in the phosphorylation of PKB (2). These findings are consistent with the hypothesis that changes in PKB activity are responsible for the changes in Ser$^{2448}$ phosphorylation observed in muscles undergoing atrophy or hypertrophy in response to changes in muscle load.

Nave et al. (20) have presented evidence that insulin and amino acid signaling converge at mTOR in HEK293 cells. The present findings in skeletal muscle are consistent with such a view, as insulin promoted the phosphorylation of Ser$^{2448}$. However, there are some differences between our results and those of Nave et al. (20). In HEK293 cells amino acid withdrawal decreased Ser$^{2448}$ phosphorylation and abolished the ability of insulin to stimulate Ser$^{2448}$ phosphorylation. In hemidiaphragms exogenous amino acids were not required for the stimulatory effect of insulin on Ser$^{2448}$ phosphorylation. Thus, there may be differences among cell types in the relative requirements for insulin and amino acids for the phosphorylation of Ser$^{2448}$. This would not be surprising as different cells exhibit differing requirements for exogenous amino acids for the stimulatory effects of insulin on the phosphorylation of PHAS-I and p70$^{S6K}$ (28, 29). However, in muscles incubated in vitro there may be adequate endogenous amino acids to allow for an insulin effect in the absence of exogenous amino acids. Amino acids did not activate PKB in skeletal muscle (Fig. 3), which is in agreement with results obtained in numerous cell types (28, 29).

In summary, the present study indicates that insulin activates PKB and promotes the phosphorylation of Ser$^{2448}$ in mTOR in skeletal muscle. Furthermore, the results demonstrate that increasing muscle load leads to increased phosphorylation of this site. The fact that these treatments increase mRNA translation (1, 2, 4), indicates that increased phosphorylation of Ser$^{2448}$ might have an important functional role and
is at the very least a marker for increased protein synthesis in skeletal muscle.

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