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mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin

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Abstract

The mTOR Complex 1 (mTORC1) protein kinase promotes growth and is the target of rapamycin, a clinically useful drug that also prolongs lifespan in model organisms. A persistent mystery is why the phosphorylation of many bona fide mTORC1 substrates is resistant to rapamycin. We find that the *in vitro* kinase activity of mTORC1 toward peptides encompassing established phosphorylation sites varies widely and correlates strongly with the resistance of the sites to rapamycin as well as to nutrient and growth factor starvation within cells. Slight modifications of the sites were sufficient to alter mTORC1 activity toward them *in vitro* and to cause concomitant changes within cells in their sensitivity to rapamycin and starvation. Thus, the intrinsic capacity of a phosphorylation site to serve as an mTORC1 substrate, a property we call substrate quality, is a major determinant of its sensitivity to modulators of the pathway. Our results reveal a mechanism through which mTORC1 effectors can respond differentially to the same signals.

Introduction

The mechanistic target of rapamycin (mTOR) is a serine-threonine kinase that serves as the catalytic subunit of two distinct signaling complexes, mTORC1 and mTORC2 (reviewed in (1)). Both are central regulators of cell growth, and mTORC1 controls key anabolic and catabolic processes in response to diverse cues, including nutrients, energy, and growth

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factors. mTORC1 is commonly deregulated in human diseases, including cancer, and therefore, there are many efforts to develop drugs that inhibit its kinase activity (reviewed in (2, 3)). The best known such drug is rapamycin, which in a complex with 12-kDa FK506-binding protein (FKBP12), binds near the mTOR kinase domain (4–7) and partially inhibits its activity (8–11). Rapamycin has drawn significant attention in the last few years not only because of its accepted clinical uses in cancer treatment and organ transplantation (1), but also for its capacity to prolong lifespan in multiple model organisms (12–17). This has led to great interest in fully understanding the mTORC1 pathway and exactly how rapamycin affects it.

Results and Discussion

Differential sensitivity of mTORC1 phosphorylation sites to rapamycin

The effects of rapamycin on mTORC1 are less straightforward than initially realized (18–20). The phosphorylation of several sites that are bona fide mTORC1 substrates, including on the key translational regulator, eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), is largely resistant to rapamycin treatment, perhaps explaining the unexpectedly weak efficacy of the drug in several early cancer clinical trials (reviewed in (21)). The differential effects of rapamycin contrast with those of ATP-competitive mTORC1 inhibitors, which block the phosphorylation of all mTORC1 phosphorylation sites regardless of their rapamycin sensitivity (18, 19, 22–24). To investigate why rapamycin differentially inhibits mTORC1 sites, we initially confirmed that this is the case under our experimental conditions in human (HEK-293E) and murine (MEF) cells. Indeed, although Torin1, a specific ATP-competitive inhibitor of mTOR (18, 25), eliminated the phosphorylation of several well-established mTORC1 sites, rapamycin affected that of only a subset of sites (Fig. 1A, B and S1).

In vitro activity of mTORC1 toward substrate phosphorylation sites predicts their rapamycin sensitivity

The extent of phosphorylation of an mTORC1 site within cells should reflect a balance between the activity of mTORC1 and of the phosphatases that dephosphorylate the site. Therefore, it is theoretically possible that certain rapamycin-resistant mTORC1 phosphorylation sites are poorly dephosphorylated so that even the reduced activity of rapamycin-bound mTORC1 is sufficient to keep them phosphorylated. Such a model predicts that rapamycin-sensitive and -resistant sites would have very different rates of dephosphorylation. To test this hypothesis, we treated cells with a dose of Torin1 that completely inhibits mTORC1 (18) and monitored the phosphorylation state of several sites over time. The results were remarkably clear: with almost identical kinetics, Torin1 caused the rapid dephosphorylation of all the sites, irrespective of their rapamycin sensitivity (Fig. 1C and D). Thus, distinct rates of dephosphorylation cannot explain the differential sensitivity of mTORC1 sites to rapamycin.

We considered the alternative possibility that it is the level of mTORC1 activity towards a particular site that determines whether it is sensitive or resistant to rapamycin within cells. Certain mTORC1 phosphorylation sites might be rapamycin resistant because they are very good substrates for mTORC1 so that even the reduced activity of rapamycin-bound mTORC1 would be sufficient to keep them phosphorylated. Relevant to this model, several mTORC1 substrates (e.g., 4E-BP1 and Grb10) have both rapamycin-sensitive and -resistant sites (Fig. 1B and S2), indicating that rapamycin sensitivity could be encoded at the level of an individual phosphorylation site rather than a full-length protein. Thus, we measured the *in vitro* kinase activity of mTORC1 towards short synthetic peptides encompassing single mTORC1 phosphorylation sites rather than towards intact proteins.

The capacity of mTORC1 to phosphorylate the peptides varied greatly and correlated strongly with the resistance of the sites to rapamycin within cells (Fig. 2A, B and S2). For example, mTORC1 weakly phosphorylated the peptide containing the rapamycin-sensitive T389 site of S6K1 but strongly phosphorylated the peptides containing the T37 or T46 sites of 4E-BP1 or S150 of Grb10, which are rapamycin-resistant. The rapamycin-sensitive sites of 4E-BP1 S65 or Grb10 S476 were, like S6K1 T389, weakly phosphorylated. We also measured mTORC1 activity towards peptides containing sites from LARP1 and PATL1, which were identified in phosphoproteomic studies as having a high likelihood of being phosphorylated by mTORC1 but are of unknown rapamycin sensitivity (26, 27). The S766 site of LARP1 turned out to be a good *in vitro* substrate, comparable to S150 of Grb10, whereas S774 was a poor one (Fig. 2C). Gratifyingly, in human cells Torin1 inhibited the phosphorylation of both sites whereas rapamycin only inhibited that of S774 (Fig. 2D). Thus, the *in vitro* activity of mTORC1 towards peptides encompassing mTORC1 phosphorylation sites can predict the rapamycin sensitivity of the sites within cells.

The rapamycin-FKBP12 complex is reported to be a partial inhibitor of mTORC1 (8–11) and indeed it attenuated the activity of mTORC1, to some extent, towards every peptide examined (Fig. 2E, bottom panel). However, it had proportionally much greater effects on sites that are poor substrates, such as T389 of S6K1 and S65 of 4E-BP1 (Fig. 2E, top panel), to the point that these sites were barely phosphorylated in the presence of the drug. Given the small size of the peptides, the differential activity of mTORC1 towards them likely reflects differences in how the peptides interact with the mTOR kinase domain rather than with other regions of mTORC1. Consistent with this notion, a truncation mutant of mTOR that retains its kinase domain but does not interact with raptor (11, 28, 29), a substrate-binding subunit of mTORC1 (30, 31), had similar peptide preferences and selectivity to those of intact mTORC1 (compare Fig. 2A with Fig. 2F; Fig. S3). In general, its *in vitro* activity was more inhibited by rapamycin than that of intact mTORC1 (compare Fig. 2E with Fig. 2G), perhaps indicating a role for raptor in stabilizing the mTOR domain kinase so that in its absence mTOR is more sensitive to allosteric inhibition by rapamycin. The binding of truncated mTOR to the peptides strongly correlated with its capacity to phosphorylate them (Fig. 2H), suggesting that mTORC1 activity towards peptide substrates is, at least in part, a consequence of their relative affinities for the mTOR kinase domain. Consistent with this possibility, rapamycin-sensitive peptides had higher K_m values than resistant peptides in steady-state kinetic analyses of mTORC1 activity (Fig. 2I). Moreover, rapamycin increased K_m values for both types of peptide substrates but had minor effects on k_{cat} values (Fig. 2I). We speculate that when FKBP12-rapamycin binds to a region adjacent to the mTOR kinase domain, it may induce a conformational change in the catalytic pocket of mTOR that reduces the accessibility of substrates. Alternatively, FKBP12-rapamycin may physically obstruct the substrate binding site, which would cause an increase in substrate K_m .

Refinement of the mTORC1 phosphorylation motif

To test if a causal relationship exists between the capacity of mTORC1 to phosphorylate a site *in vitro* and its rapamycin sensitivity within cells, it was first necessary to understand how to modify a site—defined as the phosphoacceptor serine/threonine and 4 residues on either side—to alter mTORC1 activity towards it. In a positional scanning peptide library screen mTORC1 showed a strong preference at the +1 position for proline, hydrophobic (L, V), or aromatic residues (F, W, Y) (Fig. 1B), as well as lesser selectivity at other positions, including glycine at –1 (Fig. S3) (26). The preference for both proline and non-proline hydrophobic residues in the +1 position is unusual and distinguishes mTORC1 from other proline-directed kinases such as cyclin-dependent kinases (Cdks) and mitogen-activated protein kinases (MAPKs). Using the Grb10 S150 peptide as a model substrate we confirmed

these preferences and also found that the addition of hydrophobic or charged residues promoted or decreased, respectively, mTORC1 activity towards them (Fig. 3A). In addition, substitution of the phosphoacceptor serine with threonine (Grb10 S150T) strongly reduced mTORC1 activity towards this peptide (Fig. 3A and S3). The preferences gleaned from modifying the Grb10 S150 peptide were applicable to other mTORC1 substrates. For example, elimination of glutamate and lysine from the 4E-BP1 S65 peptide boosted its phosphorylation by mTORC1 (Fig. S4), leading us to notice that poor mTORC1 phosphorylation sites tend to have several charged residues (Fig. 1C and S2). Most interestingly, however, the preference for serine over threonine as the phosphoacceptor was even stronger within the context of the S6K1 T389 peptide such that the T389S mutant was a much better mTORC1 substrate than the wild-type peptide (Fig. 3B and S5). Consistent results were obtained for serine to threonine changes in the peptides for Akt1 S473 or SGK1 S422, which are mTORC2 substrates within cells but in peptide form are phosphorylated by mTORC1 (Fig. 3B). Thus, analysis of the sequence motif specificity of mTORC1 revealed a simple way to test the hypothesis that we can increase the rapamycin resistance of a site by making it a better substrate for mTORC1.

Manipulation within cells of the rapamycin sensitivity of mTORC1 substrate phosphorylation sites

To do so we stably expressed wild-type or T389S S6K1 in MEFs lacking S6K1 and S6K2 (S6K1^{-/-}S6K2^{-/-} MEFs (32)) and monitored the effects of various treatment durations or concentrations of rapamycin on the phosphorylation of T/S389 with a phosphospecific antibody that recognizes either site equally well (Fig. S6). Upon rapamycin treatment, S389 S6K1 was dephosphorylated with slower kinetics and at higher doses than was wild-type S6K1 (Fig. 3C and D). Moreover, in mutant-expressing cells, the phosphorylation of S6 and rictor, established S6K1 substrates (33–38), was also more resistant to rapamycin (Fig. 3C and D), which is consistent with the mutant S6K1 retaining more kinase activity than its wild-type counterpart in rapamycin-treated cells (Fig. 3E). Importantly, in response to Torin1, wild-type and T389S S6K1 were dephosphorylated with very similar kinetics, indicating that the phosphatases that act on this site were unaffected by the T389S mutation (Fig. 3F). The serine mutation did not confer complete resistance to rapamycin, perhaps because it does not sufficiently increase the activity of mTORC1 toward S6K1. In addition, it is likely that the intrinsic activity of mTORC1 towards a phosphorylation site is only one of several determinants of its rapamycin sensitivity. Other properties that may have a role include the exact position of the site on the intact protein substrate, the secondary interactions the protein substrate makes with the kinase, and even perhaps its subcellular localization. Nevertheless, a single conservative change to an mTORC1 phosphorylation site is sufficient to alter its response to rapamycin and that of the downstream events the site controls.

We also tested whether making a site a poorer mTORC1 substrate increases its sensitivity to rapamycin within cells. We used ULK1, an inducer of autophagy that mTORC1 negatively regulates, in part by directly phosphorylating it on S758 (39–41). The ULK1 S758 peptide is an exceptionally good substrate *in vitro* for mTORC1, likely because it contains more than one phosphorylation site (Fig. 3G). Still, a S758T mutation was sufficient to strongly reduce the activity of mTORC1 towards the peptide (Fig. 3G and S5). Thus, we reconstituted MEFs lacking ULK1 and ULK2 (42, 43) with wild-type or the S758T ULK1 mutant and examined the extent of S/T758 phosphorylation in response to rapamycin, varying either the treatment time or dose of the drug. The phosphospecific S758 antibody recognizes phosphorylation at position 758 when either serine or threonine is the phosphoacceptor (Fig. S6). Although the phosphorylation of wild-type ULK1 was, as expected, largely resistant to rapamycin (Fig. 1A), that of the mutant was much more sensitive to rapamycin (Fig. 3H and I). Moreover, in

the mutant-expressing cells, rapamycin caused a stronger activation of autophagy, as detected by a greater decrease in p62 and a greater accumulation of LC3-II, than in cells with wild-type ULK1 (Fig. 3H, I, S7 and S8). Hence, as with S6K1, a conservative change to the ULK1 phosphorylation site is sufficient to alter its sensitivity to rapamycin as well as that of downstream signaling events, in this case autophagy induction. These results indicate that the inherent capacity of a phosphorylation site to serve as an mTORC1 substrate (its 'substrate quality') affects how it responds to the partial inhibition of mTORC1 caused by rapamycin. The same may be true for mTORC2 because phosphorylation of position 473 of Akt1 was more sensitive to low doses of Torin1 when the normal serine was changed to threonine (Fig. S9).

Substrate quality is a determinant of the mTORC1-regulated starvation program

As rapamycin is a pharmacological regulator of mTORC1, we wondered whether mTORC1 phosphorylation sites respond differentially to the physiological inputs that control mTORC1, such as nutrients and growth factors. Interestingly, we found that the same mTORC1 phosphorylation sites that were rapamycin-sensitive were also more sensitive to a partial decrease in the concentration of amino acids in the cell media. For example, the phosphorylation of T389 S6K1, which is extremely rapamycin-sensitive, was strongly reduced when cells were placed in medium with 20% of the normal levels of amino acids (Fig. 4A and S10). In contrast, the same medium did not affect the phosphorylation of S150 Grb10, which is also resistant to rapamycin, and phosphorylation of this site became partially inhibited only when cells were fully deprived of amino acids (Fig. 4A and S10). We obtained analogous results when we varied the amount of serum to which the cells were exposed or when we varied the duration of complete amino acid starvation (Fig. 4A, 4B, and S10). Thus, bona fide mTORC1 substrates vary greatly in their responses to the same mTORC1-regulating signals.

To test whether differences in substrate quality might underlie these differences in sensitivity to upstream signals, we used MEF lines expressing the wild-type or the mutant versions of S6K1 or ULK1. Phosphorylation of the T389S S6K1 mutant, which was partially resistant to rapamycin, was also strongly resistant to a reduction in amino acid concentrations and this resistance was reflected, as before, in the phosphorylation states of the S6K1 substrates S6 and rictor (Fig. 4C). Similarly, the phosphorylation of the S758T ULK1 mutant, which was sensitive to rapamycin, was also sensitive to a reduction in amino acid concentrations and to complete amino acid starvation, as were amounts of p62 and LC3-II (Fig. 4D, S11 and S12). For both kinases we obtained analogous results when we manipulated serum concentrations (Fig. S13). Thus, the substrate property that we call 'substrate quality' impacts how mTORC1 substrates respond to both pharmacological and natural regulators of the kinase. Moreover, in a competitive proliferation assay in media containing low concentrations of amino acids, the MEFs expressing T389S S6K1 outcompeted those expressing wild-type S6K1 (Fig. 4E), indicating that a change in substrate quality can also affect cell behavior.

We conclude that the sequence composition of an mTORC1 phosphorylation site, including the presence of serine or threonine as the phosphoacceptor, is one of the key determinants of whether the site is a good or poor mTORC1 substrate within cells. Even though the phosphorylation of mTORC1 sites is undoubtedly subject to varied regulatory mechanisms, we propose that differences in substrate quality are one mechanism for allowing downstream effectors of mTORC1 to respond differentially to temporal and intensity changes in the levels of nutrients and growth factors as well as pharmacological inhibitors such as rapamycin (Fig. 4F). Such differential responses are likely important for mTORC1 to coordinate and appropriately time the myriad processes that make up the vast starvation

program it controls. Lastly, it is likely that the form of hierarchical regulation we describe for mTORC1 substrates also exists in other kinase-driven signaling pathways.

Materials and Methods

Materials

Reagents were obtained from the following sources: antibodies to phospho-T389 S6K1, phospho-S235/S236 S6, phospho-T37/T46 4E-BP1, phospho-S65 4E-BP1, phospho-S70 4E-BP1, phospho-T183 PRAS40, phospho-S758 ULK1, phospho-S150 Grb10, phospho-S476 Grb10, phospho-S106 Lipin1, phospho-S472 Lipin1, phospho-S1135 Rictor, S6K1, 4E-BP1, PRAS40, FLAG, S6, and Rictor from Cell Signaling Technology; an antibody to Grb10 and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from SantaCruz Biotechnology; an antibody to p62 from Progen; antibodies to ULK1, FLAG and α -actin (clone AC-15), FLAG M2 affinity gel, ATP, FKBP12, amino acids, and insulin from Sigma-Aldrich; [γ -³²P] ATP from Perkin-Elmer; FuGENE 6, PhosSTOP, and Complete Protease Cocktail from Roche; rapamycin from LC Laboratories; DMEM from SAFC Biosciences; Inactivated Fetal Calf Serum (IFS), Fetal Bovine Serum (FBS) and SimplyBlue Coomassie G from Invitrogen; amino acid-free RPMI from US Biological; Superose 6 10/300 GL from GE Healthcare; BCA assay reagent, protein G-sepharose, streptavidin agarose, and immobilized glutathione beads from Thermo Scientific; Whatman grade P81 ion exchange chromatography paper from Fisher Scientific; QIAamp DNA Mini Kit, QuikChange XLII mutagenesis kit and XL10-Gold Competent Cells from Stratagene; SYBR Green PCR Master Mix from AB Applied Biosystems; and SAM2 Biotin Capture Membrane from Promega. Torin1 was provided by Nathanael Gray (Harvard Medical School) (18).

Cell lines and tissue culture

HEK-293E and MEFs were cultured in DMEM with 10% FBS and antibiotics. HEK-293T was cultured in DMEM with 10% IFS and antibiotics. HEK-293Es were generously provided by John Blenis (Harvard Medical School), p53^{-/-} MEFs by David Kwiatkowski (Harvard Medical School), S6K1^{+/+}S6K2^{+/+} and S6K1^{-/-}S6K2^{-/-} MEFs by Mario Pende (INSERM U845, Medical School, Paris Descartes University), ULK1^{+/+} and ULK1^{-/-} shULK2 MEFs by Reuben Shaw (Salk Institute), and ULK1^{+/+}ULK2^{+/+} and ULK1^{-/-}ULK2^{-/-} by Craig Thompson (Memorial Sloan-Kettering Cancer Center).

To generate stable cell lines, mRNA-encoding plasmids were co-transfected with Delta VPR (pLJM60/61 lentivirus) or Gag-pol envelope (pQCXIP/N retrovirus) and CMV VSV-G packaging plasmids into actively growing HEK-293T using FuGENE 6 transfection reagent as previously described (45). Virus containing supernatants were collected at 48 hr post transfection, centrifuged to eliminate floating cells, and target cells (100,000–1,000,000) infected in the presence of 8 mg/ml polybrene. 24 hr post infection, the cells were given or split into fresh media 2 μ g/mL puromycin or 1 mg/mL neomycin. mRNA-expressing cells were analyzed 2–7 days post-infection.

cDNA manipulations and mutagenesis

The mTOR truncation mutant (1295–2549) and LARP1 cDNAs were amplified by PCR, and the products were subcloned into the SalI and XhoI sites of the FLAG-tagged pQCXIP (puromycin resistant) retroviral vector for stable expression. The mLST8 cDNA was amplified by PCR, and the product was subcloned into the NotI and EcoRI sites of the pQCXIN (neomycin resistant) vector for stable expression. The S6K1 and ULK1 cDNAs were amplified by PCR, and the products were subcloned into the SalI and NotI sites of the pLJM60 (puromycin resistant) or pLJM61 (neomycin resistant) lentiviral vector for stable expression. The pLJM60 S6K1, pLJM60/61 ULK1 and pRK5 GST-tagged mouse Akt1

plasmids were mutagenized with the QuikChange XLII mutagenesis kit with oligonucleotides obtained from Integrated DNA Technologies. The S6K1, ULK1 and Akt1 mutants used in our experiments were T389S, S758T and S473T, respectively. For barcoding pLJM60 S6K1 constructs, GGATCC (BamHI) and GGTACC (KpnI) sequences were inserted in front of the start codons of wild-type and T389S S6K1, respectively, using the QuikChange XLII mutagenesis kit with oligonucleotides obtained from Integrated DNA Technologies.

Cell treatments and lysis and immunoprecipitations

For rapamycin and Torin1 treatments, 70–80% confluent cells were treated with DMSO or inhibitors as indicated in figure legends. Amino acids and serum were titrated as indicated in figure legends. Cells rinsed once with ice-cold PBS (Phosphate Buffered Saline) and lysed in ice-cold lysis buffer (50 mM HEPES pH 7.4, 40 mM NaCl, 2 mM EDTA, 1 mM orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycerophosphate, and 1% Triton X-100 or 0.3% CHAPS (for immunoprecipitations) with one PhosSTOP tablet and one tablet of EDTA-free protease inhibitors per 25 mL. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 min. For FLAG immunoprecipitations, 50% slurry of FLAG M2 affinity agarose was added to the lysates and the mixtures incubated with rotation for 2–6 hr at 4°C. Immunoprecipitates were washed three times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of sample buffer, boiled for 5 min, resolved by SDS-PAGE, and analyzed by immunoblotting as previously described (28).

Purifications of mTORC1 and truncated mTOR

mTORC1 purification from HEK-293T cells stably expressing FLAG-raptor was performed as described previously (29). Purification of the truncated mTOR mutant from HEK-293T cells stably expressing FLAG-mTOR (1295–2549) and mLST8 was also performed as described previously without a gel filtration step (29). Purified recombinant proteins were aliquoted and stored at –80°C.

In vitro kinase assays

Individual peptide substrates (GYXXXX[S/T]XXXXGRRRRR) were synthesized by the MIT Koch Institute Biopolymers and Proteomics Core Facility and purified by reversed phase HPLC. *In vitro* kinase activity of mTORC1 or truncated mTOR toward peptides was determined by incubating 0.1–0.2 mM peptide with ~100 ng mTORC1 or ~20 ng truncated mTOR in reaction buffer (25 mM HEPES pH 7.4, 50 mM KCl, 5 mM MgCl₂ and 5 mM MnCl₂) containing 50 μM cold ATP and 2–5 μCi [–32P] ATP for 20–30 min at room temperature. Aliquots (3.3 μL) of each reaction were spotted onto P81 ion exchange chromatography paper in triplicates and quenched in 0.42% H₃PO₄. Paper was washed 8–10 times in same solution and dried. Resulting radioactivity was determined by phosphorimager. For kinase assays with rapamycin, 100 nM rapamycin was preincubated with 50 ng FKBP12 for 30 min and added to reaction mixtures. FKBP12 was added in excess to ensure that most of rapamycin would be in an FKBP12-rapamycin complex.

For S6K1 kinase assays, recombinant S6K1 proteins were purified from HEK-293T stably expressing WT or T389S S6K1 using the same method as for truncated mTOR. S6 peptide substrate (AKRRRLSSLRA) was incubated in 20 μL of reaction mixture consisting of kinase assay buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂ and 5 mM MnCl₂), recombinant S6K1, 50 μM ATP and 2–5 μCi [–32P]ATP for 30 min at room temperature. Aliquots (3.3 μL) of each reaction were spotted onto P81 ion exchange chromatography paper in triplicates and quenched in 0.42% H₃PO₄. Paper was washed 8–10 times in same solution and dried. Resulting radioactivity was determined by phosphorimager.

Pull-down assay with biotinylated peptides

Biotinylated peptides were dissolved in kinase assay buffer and soluble fractions of cell lysates were collected by centrifugation at 13,000 rpm for 10 minutes. Preincubated mixtures of peptides and 50% slurry of streptavidin agarose were added to FLAG-tagged mTOR (1295–2549) and incubated in the presence of 500 nM AMP-PNP for 4–12 hr at 4°C. Pull-down mixtures were washed three times with lysis buffer containing 150 mM NaCl. Recombinant mTOR protein was denatured by addition of sample buffer, boiled for 5 min, resolved by SDS-PAGE, and analyzed by immunoblotting as previously described (28). For pull-down assays with rapamycin, 100 nM rapamycin was preincubated with 50 ng FKBP12 for 30 min and added to pull-down mixtures.

Steady-state kinetic measurements

To determine the kinetic parameters for peptide phosphorylation, assays were conducted in the presence of 40 nM mTORC1, various concentrations of peptide substrates (0, 10, 100, 250, 500 and 1000 μ) and an ATP mixture containing 500 μ M cold ATP (at least 10-fold above K_m), and 2–5 μ Ci [γ -³²P] ATP in a 30 μ L reaction mixture. The reaction was initiated via the addition of the ATP mixture. After incubation at room temperature, aliquots (3 μ L) of each reaction were spotted onto P81 ion exchange chromatography paper and quenched in 0.42% H₃PO₄. The paper was washed 8–10 times in same solution and dried. Resulting radioactivity was determined by phosphorimager. For kinetic measurements with rapamycin, 100 nM rapamycin was preincubated with 50 ng FKBP12 for 30 min and added to reaction mixtures. The steady-state kinetic parameters were obtained by fitting the reaction rates to the Michaelis-Menten equation using GraphPad Prism version 5.0 (GraphPad Inc.)

Mass Spectrometric Analyses

LARP1 phosphorylation sites were identified by mass spectrometric analysis of trypsin-digested FLAG-LARP1 purified from HEK293T cells stably overexpressing FLAG-LARP1. The amino acid positions of all LARP1 phosphorylation sites were numbered according to NCBI. Label-free quantification of LARP1 phosphorylation sites was performed with BioWorks Rev3.3 software according to the methodology previously described (31, 46).

Positional scanning peptide library screening and PWM generation

PSPL screening was performed and analyzed with the truncated mTOR mutant as previously described (47–49).

Phosphopeptide recognition by phosphospecific antibodies

1 μ L of biotinylated phosphopeptides at the indicated concentrations were spotted on a SAM2 Biotin Capture Membrane (Promega) and washed 3 times in PBST (PBS with Tween-20). Subsequently, the washed membrane was analyzed by immunoblotting as previously described (28). Phosphopeptide sequences used are as follows:

T389 S6K1: GGYFLGF[pT]YVAPGRRRRR

T389S S6K1: GGYFLGF[pS]YVAPGRRRRR

S758 ULK1: GGYFTVG[pS]PPSGGRRRRR

S758T ULK1: GGYFTVG[pT]PPSGGRRRRR

Competitive proliferation assay

S6K1^{-/-}S6K2^{-/-} MEFs stably expressing barcoded wild-type and T389S S6K1 were mixed in equal number (100,000) and placed in 10-cm culture dishes. The mixture of cells was cultured in either 100% amino acid RPMI with 10% FBS and antibiotics or 20% amino acid RPMI with 10% dialyzed FBS and antibiotics. After 32 population doublings, cells were harvested and genomic DNA was isolated using QIAamp DNA Mini Kit. The concentration and purity of DNA were determined by absorbance at 260/280 nm. Primers for real-time PCR were obtained from Integrated DNA Technologies. Reactions were run on an Applied Biosystems Prism machine using Sybr Green Master Mix (Applied Biosystems) and relative abundance of wild-type and T389S S6K1 was calculated. Primer sequences used to produce barcode-specific amplicons are as follows:

WT S6K1 forward: GTGGTGGTGCCTCGACGGGAT

WT S6K1 reverse: CACAATGTTCCATGCCAAGT

T389S S6K1 forward: GTGGTGGTGCCTCGACGGGTA

T389S S6K1 reverse: CACAATGTTCCATGCCAAGT

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Rapamycin differentially inhibits mTORC1 phosphorylation sites

(A) Responses of known mTORC1 phosphorylation sites in HEK-293E cells and mouse embryonic fibroblasts (p53^{-/-} MEFs) to 1 hr treatments with 100 nM rapamycin, 250 nM Torin1, or vehicle control. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and antibiotics. Subsequently, cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. (B) Sequence alignment of known and putative mTORC1 phosphorylation sites. Positions are numbered relative to the central phosphoacceptor serine or threonine and known rapamycin-resistant sites are indicated. (C) Dephosphorylation of mTORC1 phosphorylation sites in response to Torin1. p53^{-/-} MEFs were treated with 1 μ M Torin1, lysed at the indicated time points, and lysates analyzed as in (A). (D) Quantitation by densitometry of immunoblots shown in (A) and (C).

Figure 2. The kinase activity of mTORC1 toward peptides encompassing its phosphorylation sites correlates with their resistance to rapamycin

(A) *In vitro* kinase activity of mTORC1 toward a set of short synthetic peptides, each containing an established mTORC1 phosphorylation site, was analyzed by autoradiography (representative example shown). Phosphorylation levels of the specified peptides were quantified by densitometry. Data are means $\pm$ S.D. (n = 3 to 5). (B) *In vitro* kinase activity of mTORC1 toward indicated peptide substrates. Results are displayed as the relative kinase activity of mTORC1 for each peptide and the rapamycin sensitivity of each site is indicated. The sequences of all indicated phosphorylation sites are available in Fig. S2. (C) *In vitro* kinase activity of mTORC1 towards peptides containing indicated LARP1 sites was analyzed as in (A). (D) Rapamycin sensitivity of LARP1 phosphorylation sites in cells. FLAG immunoprecipitates from HEK-293E cells stably expressing FLAG-LARP1 and treated with 100 nM rapamycin, 250 nM Torin1 or vehicle control for 1 hr were analyzed by mass spectrometry and phosphorylation ratios determined from chromatographic peak intensities. Data are means $\pm$ S.D. (n = 3). *P < 0.05 for differences between treated and non-treated conditions (Mann-Whitney t test). (E) Effects of rapamycin-FKBP12 on mTORC1 activity towards peptide substrates. Experiment was performed and analyzed as in (A), in the presence of recombinant FKBP12 or the complex of FKBP12 and 100 nM rapamycin, using purified mTORC1 (bottom panel). Activity normalized to mTORC1 treated with FKBP12 alone for individual peptide substrates (top panel). (F) *In vitro* kinase activity of the truncation mutant of mTOR towards peptide substrates. Experiment was performed and analyzed as in (A) using the mTOR truncation mutant (amino acid positions 1295–2549) in a complex with mLST8. (G) Effects of rapamycin-FKBP12 on the activity of truncated mTOR towards peptide substrates. Experiment was performed and analyzed as in (E) using the mTOR truncation mutant. (H) Binding of the peptide substrates to the mTOR kinase domain in the absence and presence of FKBP12-rapamycin. A pull-down assay using streptavidin agarose was performed from the mixture of biotinylated peptides encompassing established mTORC1 phosphorylation sites and the FLAG-tagged mTOR truncation mutant in the presence of AMP-PNP and analyzed by immunoblotting for the FLAG tag. For pull-down assays with FKBP12-rapamycin, 100 nM rapamycin was preincubated with 50 ng FKBP12 for 30 min and added to the assay mixtures. (I) Steady-state kinetic measurements of mTORC1 kinase activity towards individual peptides encompassing mTORC1 phosphorylation sites. K_m and k_{cat} values of mTORC1 activity towards indicated peptide

substrates were determined by measuring the rate of mTORC1 phosphorylation over a range of peptide substrate concentrations (0, 10, 100, 250, 500 and 1000 μM) at a non-limiting ATP concentration, 500 μM . For kinetic measurements in the presence of FKBP12-rapamycin, 100 nM rapamycin was preincubated with 50 ng FKBP12 for 30 min and added to reaction mixtures. The steady-state kinetic parameters were obtained by fitting the reaction rates to the Michaelis-Menten equation. *Note: K_m exceeds the highest concentration tested for the S6K1 T389 peptide.

Figure 3. Conservative modifications to mTORC1 phosphorylation sites are sufficient to alter their sensitivity to rapamycin within cells

(A) *In vitro* kinase activity of mTORC1 towards peptides containing indicated modifications to the Grb10 S150 site were analyzed by autoradiography (representative example shown). Phosphorylation levels of the specified peptides were quantified by densitometry. Data are means $\pm$ S.D. (n = 3 to 5). (*P < 0.05) (B) *In vitro* kinase activity of mTORC1 toward peptides encompassing the hydrophobic motif phosphorylation sites of indicated kinases was analyzed by autoradiography as in (A). Data are means $\pm$ S.D. (*P < 0.05) (C) Time-dependent responses of wild-type and mutant T389S S6K1 to rapamycin. S6K1^{-/-}S6K2^{-/-} MEFs stably expressing wild-type or T389S S6K1 were treated with 5 nM rapamycin up to 2 hr. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Phosphorylation levels of the specified proteins were quantified by densitometry (graphs). Experiment was performed at least three times and a representative example is shown. (D) Concentration-dependent responses of wild-type and mutant S6K1 to rapamycin. S6K1^{-/-}S6K2^{-/-} MEFs stably expressing FLAG-tagged wild-type or T389S S6K1 were treated with increasing concentrations of rapamycin for 20 min and analyzed as in (C). (E) Effects of rapamycin on *in vitro* kinase activities of wild-type and mutant T389S S6K1. HEK-293T cells expressing FLAG-tagged wild-type or T389S S6K1 were treated with 50 nM rapamycin or vehicle for 15 min and the recombinant protein was purified from lysates using FLAG M2 agarose. Subsequently, *in vitro* kinase activity of wild-type or T389S S6K1 toward a S6 peptide containing the T235/236 phosphorylation sites was analyzed by autoradiography and quantified by densitometry. Data are means $\pm$ S.D. (n = 3). (*P < 0.05) (F) S6K1^{-/-}S6K2^{-/-} MEFs stably expressing wild-type or T389S S6K1 were treated with 1 μ M Torin1 for indicated time points. Cell lysates were analyzed as in (C) (representative example shown). Data are means $\pm$ S.D. (n = 3). (G) *In vitro* kinase activity of mTOR towards peptides containing indicated modifications to the ULK1 S758 site were analyzed as in (A). The high level of activity of mTORC1 towards the wild-type ULK1 peptide reflects the fact that it contains more than one site phosphorylated by mTORC1. Data are means $\pm$ S.D. (n = 3). (*P < 0.05) (H) Time-dependent responses of wild-type and mutant S758T ULK1 to rapamycin. Experiment was performed and analyzed as in (C) with ULK1^{-/-} MEFs stably expressing an shRNA against endogenous ULK2 as well as FLAG-tagged wild-type or S758T ULK1. (I) Concentration-dependent responses of wild-type and mutant S758T ULK1 to rapamycin. Experiment was performed and analyzed as in (D) for 2 hr with ULK1^{-/-}ULK2^{-/-} MEFs stably FLAG-tagged wild-type or S758T ULK1. Note: For

all peptide sequences, phosphoacceptor sites are in red text and modified residues in yellow highlight.

Figure 4. The sequence composition of mTORC1 phosphorylation sites encodes their sensitivity to physiological signals that regulate mTORC1

(A) Differential responses of established mTORC1 phosphorylation sites to partial amino acid or serum starvation. $p53^{-/-}$ MEFs were placed in media with 100, 20, or 0% of the normal levels of amino acids or 10, 2, or 0% FBS for 30 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. (B) Differential responses of established mTORC1 phosphorylation sites to complete amino acid starvation. $p53^{-/-}$ MEFs were placed in 0% amino acid media for the indicated time points. Cell lysates were analyzed as in (A). Phosphorylation levels of the specified proteins were quantified by densitometry (graph). (C) Differential concentration-dependent responses of wild-type and mutant T389S S6K1 to partial amino acid starvation. $S6K1^{-/-}S6K2^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or T389S S6K1 were placed in media with 100, 50, 20, 10, 5, or 0% of the normal levels of amino acids for 20 min. Cell lysates were analyzed as in (A). Phosphorylation levels of the specified proteins were quantified by densitometry (graph). (D) Differential concentration-dependent responses of wild-type and mutant S758T ULK1 to partial amino acid starvation. Experiment was performed and analyzed as in (C) for 2 hr under partial amino acid starvation with $ULK1^{-/-}ULK2^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or S758T ULK1. (E) A conservative change to the mTORC1 phosphorylation site S6K1 T389 is sufficient to alter the proliferation rate of cells cultured under partial amino acid starvation. $S6K1^{-/-}S6K2^{-/-}$ MEFs stably expressing barcoded wild-type and T389S S6K1 were mixed in equal number and cultured in either 100% amino acid RPMI with 10% FBS and antibiotics or 20% amino acid RPMI with 10% dialyzed FBS and antibiotics. After 32 population doublings, cells were harvested and genomic DNA was isolated and analyzed by quantitative real-time PCR. (F) Model for the role of substrate quality in the regulation of mTORC1 phosphorylation sites. Substrate quality is an important determinant of how mTORC1 substrates respond to pharmacological and natural regulators of the kinase.