Critical Role of T-Loop and H-Motif Phosphorylation in the Regulation of S6 Kinase 1 by the Tuberous Sclerosis Complex*

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The tuberous sclerosis gene products Tsc1 and Tsc2 behave as tumor suppressors by restricting cell growth, a function conserved among metazoans. Recent evidence has indicated that hyperactivation of S6 kinase 1 (S6K1) may represent an important biochemical change in the development of tuberous sclerosis-associated lesions. We show here that deletion of either Tsc1 or Tsc2 or expression of the Rheb (Ras homolog enriched in brain) GTPase leads to hyperphosphorylation of S6K1 at a subset of regulatory sites, particularly those of two essential residues functionally conserved among AGC superfamily serine/threonine kinases, i.e. the activation loop (T-loop; Thr-229) and the hydrophobic motif (H-motif; Thr-389). These sites are reciprocally and dose-dependently regulated when S6K1 is coexpressed with the Tsc1-Tsc2 complex. Mutations that render S6K1 mTOR (mammalian target of rapamycin)-resistant also protect S6K1 activity and phosphorylation from down-regulation by Tsc1/2. We demonstrate that two disease-associated mutations in Tsc2 fail to negatively regulate S6K1 activity concomitant with a failure to modify T-loop and H-motif phosphorylation. Finally, we identify one pathological Tsc2 mutation that retains its ability to negatively regulate S6K1, suggesting that, in some cases, tuberous sclerosis may develop independently of S6K1 hyperactivation. These results also highlight the importance of dual control of T-loop and H-motif phosphorylation of S6K1 by the Tsc1-Tsc2 complex.

* Tuberous sclerosis is a hyperproliferative disorder resulting in the appearance of benign tumors in multiple organ systems including brain, skin, lungs, heart, eyes, kidneys, pancreas, and the skeleton (1, 2). Initially, linkage analysis of affected families suggested that two distinct loci participated in the manifestation of the disease (3). Meanwhile, TSC1 and TSC2 were identified by positional cloning as the genes whose mutations cause tuberous sclerosis (4, 5). In metazoans, Tsc1 and Tsc2 form a signaling complex that performs a cell growth suppressive function. Studies in Drosophila have demonstrated that loss-of-function mutations in either dTsc1 or dTsc2 increase cell size and do so cell autonomously (6, 7). Conversely, overexpression of dTsc1 and dTsc2 reduces cell size, whereas expression of either product individually is without effect (6), which is consistent with a model in which the Tsc1-Tsc2 heterodimer is the functional configuration. Homozygous disruption of either Tsc1 or Tsc2 in mice is embryonically lethal, whereas mice heterozygous for either allele display increased organ size and a propensity for tumor development (8, 9).

Recently, it has been demonstrated that a deficiency in either of the tuberous sclerosis gene products leads to hyperactivation of S6K1.1 In the absence of an S6K1 crystal structure, the model of S6K1 activation is based on extensive mutational and structure-function studies and by inferences from the structure and analysis of other AGC kinases, including Akt (10, 11). It is postulated that the unphosphorylated autoinhibitory domain (AID) folds upon and occludes the amino-terminally positioned kinase domain, perhaps performing the following two functions: 1) limiting access of the substrate to the catalytic site; and 2) burying additional activating phosphorylation sites within the protein’s interior (see Fig. 1A). Activation is achieved through a coordinated and sequential series of phosphorylations beginning with phosphorylation of the carboxyl-terminal AID. Up to six serine/threonine and proline sites, designated (S/T/P), are localized to the AID and undergo phosphorylation in response to activating stimuli. Substitution of four of these sites with alanine compromises the serum-induced activation of S6K1 (12), whereas exchange of these sites with phosphomimetic acidic residues only slightly increases the basal activity (12, 13). It is therefore plausible that phosphorylation of the AID is necessary but not sufficient for S6K1 activation. Subsequently, two sites conserved among kinases of the AGC superfamily of serine/threonine kinases are phosphorylated, namely the activation loop (T-loop) site at position Thr-229 and the hydrophobic motif (H-motif) site at position Thr-389. Mutation of Thr-229 to either alanine or glutamate abolishes kinase activity (13–15). However, whereas substitution of Thr-389 with alanine abolishes kinase activity (13, 16, 17), glutamate substitution of the H-motif increases basal kinase activity (13, 16, 17). Additional phosphorylation sites have been mapped to Thr-369, Ser-371, and Ser-404 (13, 18). However, it is less clear just how the phosphorylation of these sites participates in the collective regulation of S6K1.

For S6K1, T-loop and H-motif phosphorylation is the net result of the integration of two major input pathways, nutrient sufficiency and growth factor adequacy. The nutrient sufficiency pathway senses the availability of glucose and amino acids.

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1 The abbreviations used are: S6K1, S6 kinase 1; AID, autoinhibitory domain; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; CT, carboxyl terminus; HA, hemagglutinin A; HEK, human embryonic kidney; MEF, mouse embryo fibroblast; mTOR, mammalian target of rapamycin; PDK, phosphoinositide-dependent kinase 1; P38K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Rheb, Ras homolog enriched in brain; rpS6, ribosomal protein 6.
acids as well as mitochondrial function and requires a complex comprised of mTOR, the regulatory associated protein of mTOR (Raptor), and GβL (19, 20). Raptor appears to physically recognize the TOS (target of rapamycin signaling) motif in S6K1 and another mTOR substrate, the eIF4E-binding protein (4EBP), and may function to present such substrates to the mTOR kinase (21). mTOR has shown to phosphorylate S6K1 in vitro both at the H-motif site, Thr-389, and at the AID site, Ser-411 and Thr-421/Ser-424, although Thr-389 appears to be the major site of phosphorylation (22). Small interfering RNA depletion of mTOR, Raptor, or GβL compromises nutrient stimulation of S6K1 (19), demonstrating a requirement for each component of the complex in productive signaling. It is unclear precisely how nutrient availability is sensed by the mTOR-Raptor-GβL complex. Regulation of S6K1 by the growth factor adequacy pathway involves activation of phosphatidylinositol 3-kinase (PI3K) and, thus, the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) in cellular membranes. S6K1 is activated by ectopic expression of PI3K (14) and is inhibited by the PI3K inhibitor wortmannin (12, 14), indicating that PI3K is both necessary and sufficient for S6K1 activity.

In response to growth factors, PI3K phosphorylates phosphoinositides at the D3 position of the inositol ring, producing IP3 that PI3K is both necessary and sufficient for S6K1 activity. S6K1 is activated by ectopic expression of PI3K (14) and is inhibited by the PI3K inhibitor wortmannin (12, 14), indicating that PI3K is both necessary and sufficient for S6K1 activity.

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augmented H-motif phosphorylation of S6K1 relative to wild type cells (36). It remains to be resolved, however, whether T-loop phosphorylation (Thr-229) or phosphorylation of other regulatory sites in the AID is influenced by the loss of Tsc1 or Tsc2. To address this question, S6K1 was immunoprecipitated from Tsc1- or Tsc2-null MEFs as well as from wild type MEFs under different treatment conditions. The phosphorylation status of S6K1 was assessed using antiphosphopeptide antibodies specific for the phosphorylation sites Thr-229, Ser-371, Thr-389, Ser-411, and Thr-421/Ser-424 (see Fig. 1A).

In all cell types, the pattern of basal and insulin-stimulated S6K1 activity (Fig. 1B) tightly correlated with H-motif phosphorylation at Thr-389 (Fig. 2). Importantly, whereas phosphorylation of Thr-389 in Tsc1- and Tsc2-null MEFs was inhibited by rapamycin, phosphorylation of this site was strongly resistant to inhibition by wortmannin. In contrast, S6K1 prepared from wild type MEFs displayed a reduction in H-motif phosphorylation when treated with either rapamycin or wortmannin. The wortmannin resistance of H-motif phosphorylation in MEFs devoid of Tsc1 or Tsc2 suggests that PI3K regulates S6K1 upstream of Tsc1-Tsc2 and that the effect of the loss of Tsc1-Tsc2 is dominant in the stimulation of S6K1 by PI3K. T-loop phosphorylation of S6K1 under these conditions mirrors phosphorylation of the H-motif in that this phosphorylation was largely rapamycin-sensitive and wortmannin-resistant in Tsc1- and Tsc2-null cells. A similar correlation was observed for phosphorylation of the AID sites Ser-411 and Thr-421/Ser-424. Unlike the regulation of the sites described above, the
phosphorylation of Ser-371 was neither robustly stimulated by insulin nor inhibited by rapamycin or wortmannin. Thus, phosphorylation of Ser-371 is unregulated under these conditions and appears to some extent constitutive. Analysis of the phosphorylation status of rpS6 (Fig. 2B) indicates that the cumulative effect of this multisite regulation was constitutive activation of S6K1 when Tsc1 or Tsc2 is functionally lost.

Clearly, deletion of the genes encoding Tsc1 or Tsc2 induces maximal phosphorylation of S6K1 at several regulatory sites, including the T-loop and H-motif sites. Consequently, in Tsc1- and Tsc2-null cells S6K1 is constitutively activated and refractory to conditions that would otherwise be inactivating, e.g. serum-deprivation. Overexpression of the Tsc1-Tsc2 complex in cells has the converse effect, i.e. S6K1 inhibition (28, 29, 36) (see below). We therefore sought to address whether or not this inhibition is associated with dephosphorylation of the same subset of Tsc1-Tsc2-regulated sites defined by the aforementioned studies. HEK293T cells exhibit high basal S6K1 activity (data not shown) and were therefore chosen for the subsequent experiments. Cells were transfected with increasing amounts of vectors expressing FLAG-tagged Tsc1 and FLAG-tagged Tsc2 together with HA-tagged S6K1, and S6K1 kinase activity was assayed in vitro at two different ectopic S6K1 gene dosages. Expression of Tsc1-Tsc2 led to a dosage-dependent reduction in cotransfected S6K1 activity (Fig. 3A). This effect was irrespective of the level of ectopically expressed S6K1 used in these experiments. Overexpression of Tsc1-Tsc2 reduced H-motif phosphorylation (Fig. 3B), paralleling the decrease in S6K1 kinase activity. In contrast, little change in the phosphorylation of the AID sites, Thr-421/Ser-424, was observed upon Tsc1-Tsc2 expression. This was somewhat unexpected, given the robust stimulation of Thr-421/Ser-424 phosphorylation observed in Tsc1- and Tsc2-null MEFs (Fig. 2A). Additionally, the phosphorylation of S6K1 at Ser-371 also was not significantly altered upon Tsc1-Tsc2 overexpression, which is consistent with this site remaining relatively unregulated by the Tsc1-2 complex. These data suggest that overexpression of Tsc1-Tsc2 inhibits S6K1 primarily through regulation of the H-motif and, potentially, T-loop phosphorylation, with a minimal effect on AID phosphorylation. We reasoned that the S6K1 mutations that artificially preserve or mimic H-motif phosphorylation should render the activity of the corresponding variant resistant to inhibition in cells overexpressing of Tsc1-Tsc2. Unfortunately, any modification of the T-loop site in S6K1, whether it be alanine or glutamate substitution, abolishes kinase activity (13, 14, 16). Therefore, the necessity and sufficiency of regulation of the T-loop site could not be directly tested in such an assay. A series of HA-tagged truncation and phosphomimetic S6K1 mutants were expressed in the presence or absence of FLAG-tagged Tsc1 and FLAG-tagged Tsc2, and the kinase activity of each mutant was assayed in vitro. Because the specific activities of each S6K1 construct differ as a result of each unique mutation, the activity measurements for each construct assayed in the absence of Tsc1-Tsc2 was assigned a value of 100% to allow comparison between constructs (Fig. 4A). The unadjusted kinase activity measurements are presented for reference in Fig. 4B, as is the relative expression of these constructs (Fig. 4C). Mutation of the AID, either by truncation (ΔACT) or by the acidic, phosphomimetic substitution of five serum-stimulated (S/T)P sites (D4E), failed to protect S6K1 from inhibition upon Tsc1-Tsc2 overexpression (Fig. 4A). Deletion of the amino terminus (ΔNT) of S6K1 removes the TOS motif, a small recognition/docking site found in mTOR substrates (37) that renders ΔNTΔCT rapamycin-resistant (18, 38, 39). Importantly, the activity of ΔNTΔCT was resistant to inhibition in cells in which the Tsc1-Tsc2 complex was expressed ectopically. Finally, acidic, phosphomimetic substitution of the H-motif and the AID sites (E389D3E) was also sufficient to render S6K1 resistant to inhibition by Tsc1-Tsc2. This construct has also been shown to retain 50% of its kinase activity in rapamycin-treated cells (13, 18) indicating that it, like ΔNTΔCT, is regulated, in part, independently of mTOR.

As shown in Figs. 2 and 3, the regulation of wild-type S6K1 by Tsc1-Tsc2 is achieved through control of H-motif and T-loop phosphorylation. Furthermore, phosphomimetic substitution of the H-motif is sufficient to protect S6K1 from inhibition upon the overexpression of Tsc1-Tsc2. Therefore, it is reasonable to
predict that E389D3E and ΔNTΔCT, which are resistant to inhibition by Tsc1-Tsc2, also exhibit H-motif and T-loop phosphorylation that is protected from inhibition by Tsc1-Tsc2 expression. To test this hypothesis, we compared H-motif and T-loop phosphorylation among a panel of S6K1 constructs expressed with or without Tsc1-Tsc2 in HEK293T cells. S6K1 was then immunoprecipitated with anti-HA antibody and immunoblotted using phospho-specific antisera. Whereas the phosphorylation of Thr-229 and Thr-389 in wild type and ΔNTΔCT S6K1 was reduced in cells coexpressing Tsc1-Tsc2, neither T-loop nor H-motif phosphorylation was affected by overexpressed Tsc1-Tsc2 in the E389D3E or ΔNTΔCT constructs (Fig. 5A) (note that the anti-phospho-Thr-389 antibody used in these studies recognizes the S6K1 mutant with a glutamate substitution at position 389). These constructs were expressed at similar levels as shown by analysis of whole cell lysates (Fig. 5B). Collectively, these data argue that the inhibition of S6K1 observed in cells expressing Tsc1-Tsc2 is due to specific inhibition of the H-motif phosphorylation.
overexpressing Tsc1-Tsc2 reflects both reduced T-loop and H-motif phosphorylation.

Presumably, disease-associated mutations in Tsc1 and Tsc2 result in the loss of some essential function of the complex. If S6K1 hyperactivation is important for the progression of tuberous sclerosis, those mutations causative for the development of tuberous sclerosis should also show defective regulation of S6K1. To test this possibility, HA-tagged S6K1 was coexpressed with FLAG-tagged Tsc1 alone or in combination with a panel of naturally occurring, disease-associated FLAG-tagged Tsc2 mutants in HEK293T cells. S6K1 was immunoprecipitated, and its kinase activity and the extent of T-loop and H-motif phosphorylation were determined. In this assay, little change in either the kinase activity (Fig. 6A) or in the phosphorylation of the T-loop or the H-motif (Fig. 6C) was observed when Tsc1 or Tsc2 was expressed alone. However, coexpression of Tsc1 and Tsc2 was sufficient to reduce both S6K1 activity as well as T-loop and H-motif phosphorylation, supporting the idea that Tsc1 and Tsc2 are each critical components of the signaling complex. Of the disease-associated Tsc2 substitutions tested in this assay, F615S and Y1571H failed to regulate S6K1 in regard to both kinase activity and T-loop and H-motif phosphorylation. However, N525S Tsc2 was competent to negatively regulate S6K1, as evidenced both by the reduction in coexpressed S6K1 kinase activity and phosphorylation. These differences were not due to variations in the expression level of the Tsc2 constructs (Fig. 6C).

Coexpression of the Rheb GTPase is sufficient to induce S6K1 activation concomitant with H-motif phosphorylation (32). We therefore coexpressed HA-tagged S6K1 with or without Myc-tagged Rheb in HEK293 cells, which, unlike HEK293T cells, have low basal S6K1 activity when serum starved, and we immunoprecipitated S6K1 with anti-HA antibodies. As shown in Fig. 6D, the expression of Rheb induced phosphorylation of both the T-loop and the H-motif. The expression levels of Rheb and S6K1 in whole cell lysates are also shown (Fig. 6E).

**DISCUSSION**

In this study we have demonstrated that the T-loop and H-motif are primary sites of phosphorylation-dependent regulation of S6K1 by Tsc1-Tsc2. Although Ser-371 is necessary for kinase activation (40), in MEFs the phosphorylation of this site is unaffected by the presence of insulin or by the inhibitors wortmannin or rapamycin (Fig. 2A). Furthermore, Ser-371 phosphorylation was neither significantly regulated by the loss of Tsc1 or Tsc2 (Fig. 2A) nor by overexpression of the Tsc1-Tsc2 complex (Fig. 3B). Loss of either Tsc1 or Tsc2 resulted in increased basal AID phosphorylation at Ser-411, whereas the phosphorylation of the adjacent AID sites Thr-421/Ser-424 was similar to wild type levels (Fig. 2A). Collectively, these sites were sensitive to wortmannin and rapamycin in wild type MEFs, whereas the phosphorylation of these sites were wortmannin-insensitive in Tsc1- or Tsc2-null MEFs. Similar to the phosphorylation of Ser-371, Thr-421/Ser-424 was essentially unregulated in HEK293T cells overexpressing Tsc1-Tsc2 (Fig. 3B). Therefore, the reduction in S6K1 activity associated with overexpression of Tsc1-Tsc2 is less dependent on the regulation of Ser-371 and Thr-421/Ser-424 phosphorylation and, instead, tightly correlates with the regulation of the T-loop and H-motif sites.

How then is the regulation of S6K1 by Tsc1-Tsc2 achieved? In Tsc1- and Tsc2-null MEFs, the T-loop site (Thr-229) and the H-motif site (Thr-389) are constitutively phosphorylated to the levels observed in wild type MEFs stimulated with insulin (Fig. 2A). Similarly, overexpression of Rheb induces both T-loop and H-motif phosphorylation (Fig. 6D). Conversely, the phosphorylation of these sites is reduced in cells overexpressing the Tsc1-Tsc2 complex (Figs. 3B, 5A, and 6C). Biondi and colleagues (27) have demonstrated that, in vitro, a carboxyl-terminally truncated S6K1 is a much better PDK1 substrate when the S6K1 H-motif surrogate phosphopeptide, suggesting that PDK1 interacts with the phosphorylated H-motif of S6K1 and that this event increases the efficiency with which PDK1 phosphorylates the T-loop of S6K1. Given that the kinase activity of PDK1 is not increased by growth factors or stimuli that induce PI3K...
activation (24, 41), and given that PDK1 and S6K1 are constitutively associated when expressed in cells (42), it is plausible that loss of Tsc1 or Tsc2, through activation of Rheb, induces H-motif phosphorylation. The phosphorylated H-motif is then recognized by PDK1, which facilitates T-loop phosphorylation of S6K1. Given the importance of T-loop and H-motif phosphorylation in modulating the catalytic activity of AGC kinases, the coordinated regulation of these motifs in S6K1 explains the reciprocal regulation of S6K1 catalytic activity in cells that lack or overexpress Tsc1/2. Furthermore, the sensitivity of both S6K1 activity and T-loop and H-motif phosphorylation to rapamycin indicates that mTOR signaling activity is required for hyperactivation of S6K1 in Tsc1- and Tsc2-deficient MEFs. Conversely, the wortmannin-resistance of S6K1 activity and phosphorylation in these cells demonstrates that the Tsc1/Tsc2 deficiency induces S6K1 activation independently of PI3K.

Independent genetic screens of tuberous sclerosis patients have identified dozens of disease-associated lesions spanning virtually the entire length of the Tsc2 protein (43–48). Several of these mutations, e.g. F615S and Y1571H, abrogate not only the interaction with Tsc1 but also the tyrosine phosphorylation of Tsc2 (49, 50), potentially explaining the loss of function of the respective Tsc2 variant. Although Tsc2 is tyrosine-phosphorylated in response to mitogenic stimuli (50), the function of this phosphorylation is unclear. However, the Tsc2 mutation N525S affects neither Tsc1 interaction nor tyrosine phosphorylation of Tsc2 (49), suggesting that the manifestation of tuberous sclerosis may involve biochemical alterations other than Tsc1-Tsc2 interaction and Tsc2 tyrosine phosphorylation. As shown in Fig. 6, A–C, there is a strong correlation between S6K1 regulation and the reported biochemical properties described above, i.e. interaction with Tsc1 and Tsc2 tyrosine phosphorylation. It would therefore appear that regulation of S6K1 involves productive assembly of the Tsc1-Tsc2 complex and involves Tsc2 tyrosine phosphorylation. The observation that N525S negatively regulates S6K1 as well as wild type Tsc2 raises the intriguing possibility that the development of tuberous sclerosis may, in some cases, occur independently of S6K1 hyperactivation. It would therefore be interesting to determine whether or not tuberous sclerosis-associated lesions harboring...
the N525S mutation in Tsc2 exhibit normal S6K1 activation. Inhibition of the mTOR signaling pathway is rapidly emerging as a promising strategy for the treatment of solid tumors, particularly those resulting from constitutive activation of the PI3K/Akt pathway, such as PTEN-inactivating mutations (51–55). Albeit largely benign, tuberous sclerosis-associated tumors can influence almost every major organ system and are thus responsible for the wide spectrum of physiological complications in affected individuals. The observations that mTOR signaling is up-regulated in these lesions suggest that rapamycin or its second generation derivatives may prove effective in the treatment of tuberous sclerosis. Our results, however, indicate that not all lesions resulting from Tsc2 mutation may be equally responsive to such a therapeutic approach.

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