The tuberous sclerosis complex (TSC)\(^*\) is a genetic disorder that leads to the development of benign hamartoma-tous tumors in the brain, kidneys, heart, eyes, lungs, and skin. The disorder affects about 1 million individuals worldwide, with an estimated prevalence of one in 6,000 newborns. Common symptoms include seizures, mental retardation, behavior problems, and skin abnormalities (for review see Ref. 1). Genetic studies have mapped this disorder to the \(\text{TSC1}\) and \(\text{TSC2}\) genes that encode the protein products hamartin \((-130\, \text{kDa})\) and tuberin \((-200\, \text{kDa})\), respectively.

Hamartin and tuberin negatively regulate cell growth and proliferation as a tuberin-hamartin heterodimer \((2, 3)\). \textit{Drosophila} and mouse genetics show that mutations in \(\text{TSC1}\) or \(\text{TSC2}\) result in increased cell size \((\text{an increase in cell mass/cell growth})\) and cellular proliferation \((\text{an increase in cell number})\) \((4–6)\). Genetic epistasis analysis in \textit{Drosophila} places \(\text{dTSC1/dTSC2}\) downstream of \(\text{dPI3K}\) and \(\text{dAkt}\) (also referred to as protein kinase \(\text{B}\)) but upstream of \textit{Drosophila} ribosomal protein S6 kinase \(\text{dS6K}\) \((6–7)\). The loss of function or overexpression of several \textit{Drosophila} genes recently linked the \(\text{dPI3K}\) and \(\text{dTOR}\) signaling pathways to the regulation of cell proliferation and cell growth \((8)\), important cellular processes that are inappropriately regulated during tumor formation. These include \(\text{dPI3K}\), \(\text{dAkt}\), \(\text{dS6K}\), and \(\text{dE-BP}\) (effectors of \(\text{dPI3K}\)), and \(\text{dTOR}\). Collectively, this work suggested that \(\text{dTOR}\) and \(\text{dTSC1/dTSC2}\) were connected via the \(\text{PI3K}\) signaling pathway.

Recently, a surge of research from many labs revealed that \(\text{TSC1/TSC2}\) functioned to inhibit nutrient- and \(\text{PI3K}\)-dependent signaling through \(\text{mTOR}\) (also called FRAP or RAFT), resulting in the inhibition of its downstream targets \(\text{S6K1}\) and \(\text{E-BP1}\) \((9–12)\). Although the majority of work supports this notion, the \(\text{TSC1/TSC2}\) may also function in some circumstances to inhibit \(\text{PI3K}\)-dependent signaling to \(\text{S6K1}\) independently of \(\text{mTOR}\) (see Ref. 13 and for review see Ref. 14). It is clear, however, through the use of different experimental approaches, that \(\text{PI3K}\)-dependent signaling serves to inhibit the tuberin-hamartin heterodimer through direct Akt-dependent phosphorylation of tuberin at Ser-939 and Thr-1462 \((15)\). Together, these observations suggest that the tuberin-hamartin heterodimer inhibits \(\text{mTOR}\) and that Akt can phosphorylate tuberin, thereby inactivating it.

\(\text{mTOR}\) is regulated by mitogens, nutrients, and energy availability and is a critical upstream regulator of \(\text{S6K1}\) and \(\text{E-BP1}\) for reviews see Ref. 16). The \(\text{mTOR}\) inhibitor, rapamycin, induces rapid \(\text{S6K1}\) and \(\text{E-BP1}\) dephosphorylation. Regulation of \(\text{S6K1}\) and \(\text{E-BP1}\) phosphorylation by stimulation of two distinct signaling pathways, \(\text{PI3K}\) and \(\text{MAPK}\), require an \(\text{mTOR}\) input, as rapamycin can potently inhibit the phosphorylation of these downstream effectors by both \(\text{PI3K}\) and \(\text{MAPK}\)-regulated pathways \((16–18)\). Hypophosphorylated \(\text{E-BP1}\) binds to the cap-binding protein \(\text{eIF4E}\) to inhibit cap-dependent translation (for review see Ref. 19), which is reversed when \(\text{E-BP1}\) is phosphorylated. The dissociation of hyperphosphorylated \(\text{E-BP1}\) from \(\text{eIF4E}\) leads to the binding of \(\text{eIF4G}\) to \(\text{eIF4E}\) and the initiation of protein synthesis. It has been shown that both \(\text{S6K1}\) and \(\text{eIF4E/4E-BP1}\) effect \(\text{mTOR}\)-dependent control of mammalian cell size \((20)\). The research summarized above, combined with the \textit{Drosophila} genetic studies \((8)\), strongly imply that aberrantly high \(\text{mTOR}\)-dependent

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signaling to both S6K1 and eIF4E/4E-BP1 contributes to the expansion and size of TSC tumors.

It has been known for some time that phosphorylation of S6K1 and 4E-BP1 are regulated by PI3K and/or by PKC-dependent (phorbol ester-activated) pathways (21–24). We use phorbol 12-myristate 13-acetate (PMA), a phorbol ester and a tumor promoter, to induce S6K1 and 4E-BP1 phosphorylation via activation of the conventional and novel PKCs. In this report we show that the TSC1/TSC2 inhibits PKC/MAPK signaling toward 4E-BP1 and S6K1. Because PMA does not activate PI3K-dependent signaling in the HEK293E cells used in this study, these data support the model that TSC1/TSC2 inhibits mTOR rather than PI3K-mediated signaling. We report that tuberin is phosphorylated on the Akt-dependent sites (Ser-939 and Thr-1462) and PKC (bisindolylmaleimide I). This study reveals that the PKC/MAPK signaling input to tuberin is distinct from that mediated by PI3K/Akt. Furthermore, our work addresses an important question as to how S6K1 is activated upon PI3K-dependent signaling. Our findings provide evidence that cell signaling through converging PI3K and PKC/MAPK pathways inactivate the tuberin-hamartin heterodimer as a result of tuberin phosphorylation at overlapping sites.

MATERIALS AND METHODS

Chemicals and Materials—Wortmannin and U0126 were purchased from Biomol (Plymouth Meeting, PA), and bisindolylmaleimide I was from Calbiochem. PMA, insulin, and anisomycin were bought from Sigma, and epidermal growth factor (EGF) was from Invitrogen. PA was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Radiolabeled reagents were purchased from PerkinElmer Life Sciences, and m7GTP-Sepharose was from Amersham Biosciences. All other reagents (unless stated) were obtained from VWR Scientific (West Chester, PA).

Plasmids—N-terminal FLAG-tagged pRK7/TSC1 and pRK7/TSC2 mammalian expression vectors were generated as described previously (12). The N-terminal FLAG-tagged tuberin double point mutant (Ser-939 and Thr-1462 to Ala) cDNA, which was generously provided by B. D. Manning and L. C. Cantley (Harvard University, Boston), was subcloned into pRK7. pACTAG2 expressing human HA-tagged TSC1 was a kind gift from M. Chou (University of Pennsylvania, Philadelphia), and anti-4E-BP1, -tuberin (Thr-1462 phospho-specific), -Akt, and -eIF4E antibodies were bought from Cell Signaling Technology (Beverly, MA). The C-90 anti-tuberin antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-p38MAPK antibodies were purchased from R & D Systems Inc. (Minneapolis, MN). Anti-MAPK antibodies were generated as described previously (28). eIF4E was purified by using affinity chromatography on m7GTP-Sepharose as described (20). For analysis of hamartin and tuberin within the insoluble pellet, the cell lysates were prepared as described previously (12).

Immunoprecipitation and Immune Complex Kinase Assays—For immunoprecipitation studies of HA-tagged S6K1, cell extracts were immunoprecipitated with anti-HA antibodies bound to protein A-Sepharose (Pharmacia Corp., Peapack, NJ) for 3 h. Immunoprecipitates were washed as described previously (25). S6K1 kinase activity was determined in vitro by using recombinant GST-S6 (22 C-terminal amino acids of ribosomal protein S6) as a substrate, as described previously (25). Quantification of incorporation of the 32P label was quantified on a Bio-Rad PhosphoImager with ImageQuant software. In vivo 32P radiolabeling of tuberin was carried out in serum-starved HEK293E cells overexpressing hamartin and tuberin. The cells were incubated in phosphate-free medium for 4 h, and the cells were treated and pulsed with 5 μCi of [32P]orthophosphate for 2 h. FLAG-tagged hamartin and tuberin were immunoprecipitated with an anti-FLAG antibody bound to protein G-Sepharose (Pharmacia Corp.) for 1 h. Immunoprecipitates were washed as for the S6K1 kinase assays (see above) and then subjected to SDS-PAGE and autoradiography.

RESULTS

Co-expression of Hamartin and Tuberin Impairs PMA-induced 4E-BP1 Phosphorylation and eIF4E Function Independently of PI3K—Previously, we reported that co-expression of both hamartin and tuberin blocked 4E-BP1 phosphorylation upon insulin stimulation of HEK293E cells (12). In this cell line, insulin strongly stimulates signaling through the PI3K pathway but not the MAPK pathway (12). Given that activation of the MAPK pathway by phorbol esters results in phosphorylation of 4E-BP1 that is dependent on mTOR but independent of PI3K (23, 24), we examined whether hamartin and/or tuberin could affect PMA-induced 4E-BP1 phosphorylation.

Hamartin and tuberin were co-expressed with 4E-BP1 in HEK293E cells that were serum-starved and then stimulated with PMA. By using insulin as a control to stimulate PI3K-dependent signaling, we show that PMA stimulation of this cell line activates the PKC/MAPK signaling pathway but not the PI3K/Akt pathway, as shown by the ability of PMA to stimulate phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (also known as MAPK) without inducing phosphorylation of Akt on Thr-308 (Fig. 1A). The level of hamartin and tuberin within the cell lysates were compared (Fig. 1B) and revealed that the amount of hamartin and tuberin protein was enhanced when each was co-expressed with the other, as reported previously (12, 29). The ability of hamartin and tuberin to impair PMA-induced 4E-BP1 phosphorylation was analyzed by a gel shift assay in conjunction with phospho-specific antibodies that recognize 4E-BP1 when phosphorylated on Thr-37/Thr-46, Ser-65, and Thr-70. The α-, β-, and γ-isoforms of 4E-BP1 resolve as three separate bands on high acrylamide SDS gels. The lowest α-isoform band and the highest γ-isoform band contain the least and most phosphorylated species of 4E-BP1, respectively.

overexpression of tuberin without its binding partner, hamartin, partially impaired the phosphorylation of 4E-BP1, which was further enhanced when hamartin was co-expressed with tuberin. Hamartin and tuberin co-expression inhibited the “priming” phosphorylation of 4E-BP1 at Thr-37/46 upon PMA stimulation, similar to the effect of rapamycin. Phosphorylation of Thr-37/46 is necessary for the optimal phosphorylation of the latter sites Thr-70 and Ser-65 (for review see Ref. 19). Ser-65 and Thr-70 phosphorylations are required for the dissociation of 4E-BP1 from eIF4E (reviewed in Ref. 19), where the occurrence of Ser-65 phosphorylation coincides with its...
Patients Are Unable to Repress PMA-induced 4E-BP1 Phosphorylation. HEK293E cells transiently transfected with plasmids expressing FLAG-tagged hamartin and tuberin (Ham and Tub, respectively), where indicated, were serum-starved, pre-treated with 25 nM rapamycin (Rap) for 30 min, and then stimulated with 100 ng/ml PMA for 40 min, where indicated. Previous work showed that PMA-induced PKC activation of Akt was fully blocked by the PKC inhibitor bisindolylmaleimide I (Fig. 3). PMA potently activated S6K1, which was fully blocked by 5 μM bisindolylmaleimide I, a PKC inhibitor that abolished the PMA-induced PKC activation of ERK1/2 (as observed by bisindolylmaleimide I to prevent ERK1/2 phosphorylation). Under conditions where wortman-...
Tuberin and Hamartin Overexpression Inhibit S6K1 Activity Independently of PI3K—To confirm whether hamartin and tuberin co-expression inhibits S6K1 via a PI3K-independent mechanism, it was necessary to activate S6K1 in the complete absence of PI3K signaling. To do this, we stimulated HEK293E cells with either EGF or PA in the presence of wortmannin (to completely impair basal signaling through PI3K) (Fig. 4). Given that wortmannin blocked insulin-induced Thr-308 phosphorylation of Akt, the EGF- or PA-induced activation of S6K1 in the presence of wortmannin occurs independently of PI3K. EGF-induced activation of S6K1 was blocked by both rapamycin and U0126 (Fig. 4A, lanes 5 and 6, respectively) and therefore shows a requirement for both mTOR and MEK to activate S6K1 in the absence of PI3K-mediated signaling. Considering that bisindolylmaleimide I did not prevent ERK1/2 phosphorylation or EGF-induced S6K1 activation, whereas the U0126 compound did (Fig. 4A, lanes 7 and 6, respectively), suggests that the PI3K-independent increased activity of S6K1 by EGF must be largely dependent on activation of MAPK signaling rather than PKC signaling. Furthermore, this experiment confirms that the concentration of bisindolylmaleimide I used does not inhibit MAPK signaling to ERK1/2 or S6K1 non-specifically. Hamartin and tuberin co-expression blocked the PI3K-independent activation of S6K1 upon EGF stimulation by ~70% (Fig. 4A, lane 8), which was repressed further upon pre-treatment with the U0126 compound (Fig. 4A, lane 9). PMA treatment that activates the PKC/MAPK pathway potently stimulated S6K1 activity 19-fold (Fig. 4A, lane 11), which was fully blocked by rapamycin (Fig. 4A, lane 12) and markedly impaired by either U0126 or bisindolylmaleimide I (Fig. 4A, lanes 13 and 14, respectively), treatments that were sufficient to block ERK1/2 phosphorylation upon PMA treatment. Hamartin and tuberin co-expression repressed the PI3K-independent PMA activation of S6K1 by ~80% (Fig. 4A, lane 15), which was reduced to levels lower than that of the basal with either U0126 or bisindolylmaleimide I (Fig. 4A, lanes 16 and 17, respectively).

We show that hamartin and tuberin co-expression in conjunction with U0126 treatment significantly inhibited EGF- and PMA-induced activation of S6K1 more than either hamartin/tuberin co-expression or U0126 treatment alone (see Fig. 4B), suggesting that tuberin-hamartin inhibits signaling through mTOR. Importantly, wortmannin was used in conjunction with either EGF or PMA treatments revealing that hamartin and tuberin represses S6K1 activity independently of PI3K.

Phosphatidic Acid (PA)-mediated mTOR Signaling Is Impaired by Hamartin and Tuberin Co-expression—To further confirm whether tuberin-hamartin inhibits mTOR-dependent signaling, we pre-treated HEK293E cells with wortmannin to inhibit basal PI3K-mediated signaling while promoting mTOR signaling with PA. PA has been shown to activate mTOR signaling through the direct binding of PA to the PKB12rapamycin-binding domain of mTOR (31). Co-expression of hamartin and tuberin impaired the activation of S6K1 upon PA treatment (Fig. 5). As reported previously (31), PA did not stimulate either Akt or ERK1/2 phosphorylation, indicating that S6K1 activation upon PA treatment was a result of increased mTOR signaling toward S6K1. Therefore, these data further support the idea that the tuberin-hamartin directly inhibits mTOR-dependent signaling.

PKC/MAPK-mediated In Vivo Tuberin Phosphorylation Occurs Independently of PI3K/Akt—The ability of PMA to activate S6K1 independently of PI3K suggests that a PI3K-independent input must inhibit the tuberin-hamartin heterodimer so that S6K1 can become activated by PKC/MAPK signaling. Because the function of tuberin-hamartin is modulated through Akt-dependent phosphorylation of tuberin (9, 10, 15, 32), we wanted to investigate whether endogenous tuberin was also phosphorylated upon activation of the PKC/MAPK signaling pathway under conditions where Akt was inactive (Fig. 6A). Phosphorylation of tuberin retards its mobility on SDS-PAGE, as observed when HEK293E cells were treated with PA (Fig. 6A, upper panel). Phosphorylation of tuberin by PA was either impaired or fully prevented by pre-treating the cells with U0126 and bisindolylmaleimide I, respectively. The PI3K-induced phosphorylation of tuberin was unaffected by wortmannin and reveals that both PKC and MAPK signaling leads to the phosphorylation of tuberin in the absence of PI3K signaling. Interestingly, insulin treatment, which resulted in increase reactivity of tuberin with both anti-phospho-Thr-1462 and anti-phospho-Akt substrate antibodies (Fig. 6A, 2nd and 3rd panel from top, respectively), did not alter the mobility of tuberin on SDS-PAGE. The anti-phospho-Akt substrate antibody recognizes proteins that are phosphorylated on the Ser or Thr within the RRXX(S/T) motif (where X is any amino acid). This anti-phospho-Akt substrate antibody was first used to show that Akt phosphorylated tuberin on Ser-939 and Thr-1462 and that both conform to the RRXX(S/T) consensus sequence (9). Therefore, the observed mobility shift of tuberin...
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upon PMA treatment likely occurs through the phosphorylation of other as yet unidentified sites that are distinct from those phosphorylated by insulin-dependent Akt activation. Surprisingly, PMA treatment caused an increase in tuberin phosphorylation at the Akt consensus phosphorylation RXRXX(S/T) sites (as observed by the increased detection of tuberin with the Akt substrate antibody (Fig. 6A, 2nd panel from top)) that can be partially attributed to phosphorylation of tuberin at Thr-1462 (Fig. 6A, 3rd panel from top). Detection of PMA-induced phosphorylation with the anti-Akt substrate an-

Fig. 4. Tuberin-hamartin complexes inhibit S6K1 activity independently of PI3K. HEK293E cells were transiently transfected with plasmids expressing FLAG-tagged hamartin and tuberin (Ham/Tub), where indicated, in conjunction with HA-tagged S6K1. The cells were serum-starved, pre-treated with either 100 nM wortmannin (WORT), 25 nM rapamycin (RAP), 25 μM U0126, or 5 μM bisindolylmaleimide I (BIM) for 30 min and then stimulated with either 100 nM insulin (Ins), 25 ng/ml EGF, or 100 ng/ml PMA for 30, 10, or 40 min, respectively, where indicated. A, protein levels of hamartin, tuberin, ERK1/2, and Akt and levels of phosphorylation of ERK1/2 and Akt were determined as in Fig. 1. S6K1 activity assays were carried out as described under “Materials and Methods.” The levels of S6K1 were determined with the α-HA antibody (S6K1(α-HA)). Incorporation of 32P label into the GST-S6 substrate was quantified using a PhosphorImager, and an autoradiograph of the gel is shown (bottom panel). The lanes are numbered 1–17. B, the fold activation of S6K1 upon treatment with EGF (top graph) and PMA (bottom graph) in the presence of wortmannin is graphed.

Fig. 5. Tuberin and hamartin overexpression impairs PA-mediated mTOR signaling. HEK293E cells were transiently transfected with HA-tagged S6K1 and FLAG-tagged hamartin and tuberin, where indicated. These cells were then serum-starved, pre-treated with 100 nM wortmannin (Wort) in the presence or absence of 25 μM rapamycin (Rap) for 30 min, where indicated, and then stimulated with either serum (10% fetal bovine serum) or 100 μg/ml PA for 30 min. Protein levels of hamartin (Ham), tuberin (Tub), ERK1/2, and Akt and levels of phosphorylation of ERK1/2 and Akt were determined as in Fig. 1. S6K1 activity assays were carried out as described under “Materials and Methods.” The levels of S6K1 were determined with the α-HA antibody (S6K1(α-HA)). Incorporation of 32P label into the GST-S6 substrate was quantified using a PhosphorImager, and an autoradiograph of the gel is shown (bottom panel).
tobody was insensitive to wortmannin but inhibited by both U0126 and bisindolylmaleimide I, revealing that Akt is not the kinase involved in the phosphorylation of tuberin at Thr-1462 or the RXX(S/T) consensus sites upon PMA treatment. This finding reveals that tuberin is phosphorylated in vivo by a protein kinase regulated by the PKC/MAPK signaling pathway that recognizes a similar consensus phosphorylation motif to that of Akt.

To examine the extent of tuberin phosphorylation upon PMA treatment in more detail, we radiolabeled exogenous tuberin in vivo. To confirm whether phosphorylation of tuberin by PMA occurred in the absence of basal PI3K/Akt signaling, we compared the incorporation of radiolabeled phosphate into FLAG-tagged tuberin upon PMA treatment in the presence or absence of U0126 or bisindolylmaleimide I, in conjunction with wortmannin (Fig. 6B). To facilitate good incorporation of radiolabeled phosphate into tuberin, hamartin was co-expressed with tuberin to increase the levels of the tuberin-hamartin heterodimer within the cells. PMA markedly enhanced the phosphorylation of tuberin, which was blocked by pre-treating the cells with either U0126 or bisindolylmaleimide I. Interestingly, there was no significant increase of $^{32}$P-radiolabeled incorporation into hamartin upon PMA treatment, revealing that the PKC/MAPK signaling pathway modulates the function of the tuberin-hamartin heterodimer by phosphorylating tuberin rather than hamartin.

The data presented in Fig. 6A suggest that the phosphorylation of tuberin upon PMA treatment occurs at sites that overlap with and are unique to those that are phosphorylated by Akt. To address this possibility further, we measured $^{32}$P-radiolabeled incorporation into a double point mutant of tuberin, which has the two main Akt phosphorylation sites (Ser-939 and Thr-1462) mutated to alanines (referred to as “tuberin(SATA)”), upon PMA and insulin stimulation (Fig. 6C). As expected, insulin stimulation, which activated Akt but not ERK1/2, enhanced the incorporation of $^{32}$P radiolabel into wild-type tuberin but not the tuberin(S939A/T1462A) mutant that lacked the Akt phosphorylation sites. Confirming the notion that tuberin can be phosphorylated at Akt-independent sites, PMA treatment enhanced the incorporation of $^{32}$P radiolabel into the wild-type and the mutant (S939A/T1462A) tuberin protein. PMA-induced phosphorylation of the tuberin(S939A/T1462A) mutant was blocked by pre-treatment with either U0126 or bisindolylmaleimide I, cell treatments that were sufficient to prevent phosphorylation of ERK1/2. These data indicate that two independent signaling pathways, PI3K/Akt and PKC/MAPK, lead to the phosphorylation of overlapping and distinct sites on tuberin.
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**Tubulin(S939A/T1462A) Mutant Impairs S6K1 Activity**—To investigate whether tuberin phosphorylation at Ser-939 and Thr-1462 inactivates the function of the protein upon PMA stimulation, we compared the effects of wild-type tuberin and tubulin(S939A/T1462A) to block PMA-induced S6K1 activation. We observed that the S939A/T1462A tuberin mutant was more effective at inhibiting S6K1 activation after stimulation with PMA than the wild-type tuberin (Fig. 7). The S939A/T1462A tuberin mutant inhibited S6K1 activity by 70%, whereas the wild-type only inhibited S6K1 by 35%. This finding indicates that tuberin phosphorylation at Ser-939 and Thr-1462 through the PKC/MAPK-mediated pathway functions to inhibit tuberin.

**PMA-induced Tuberin Phosphorylation Is Independent of p38MAPK**—The p38MAPK-activated kinase MK2 (also referred to as MAPKAPK2) has been shown to phosphorylate tuberin on Ser-1210 (33). It should be noted that the MK2 phosphorylation site within tuberin is “LYKSLS” (where the last amino acid is Ser-1210), which does not conform to the consensus RRXX(S/T) sequence. Therefore, it is unlikely that the anti-phospho-Akt substrate antibody would recognize tuberin when phosphorylated on Ser-1210. To determine whether activation of the p38MAPK/MK2 signaling pathway promotes tuberin phosphorylation on the Akt consensus RRXX(S/T) sites, we stimulated cells with anisomycin (referred to as “Ans”), a stress agonist (Fig. 8). Cell treatments with anisomycin induced p38MAPK phosphorylation but did not result in tuberin phosphorylation on the RRXX(S/T) motifs. In contrast, insulin and PMA treatments induced tuberin phosphorylation on the RRXX(S/T) sites that were blocked by treatments with wortmannin (to block PI3K) or bisindolylmaleimide I (to block PKC), respectively. Therefore, activation of the p38MAPK/MK2 signaling pathway does not induce tuberin phosphorylation on the RRXX(S/T) sites that are phosphorylated upon activation of either the PI3K/Akt or PKC/MAPK signaling pathways.

**DISCUSSION**

Previous work (9–13) clearly depicts that the TSC1/2 inhibits PI3K/Akt-mediated signaling toward S6K1 and 4E-BP1. There has been some controversy, however, as to whether tuberin-hamartin inhibits PI3K signaling through an mTOR-dependent or -independent mechanism (for review see Ref. 14). The data presented here strongly argue that mTOR-dependent signaling is indeed the target of TSC1/2. We report that tuberin-hamartin inhibits 4E-BP1 phosphorylation (Figs. 1B and 2) and S6K1 activity (Figs. 3, 4, A and B, and 5) upon cell treatments that activate the mTOR signaling pathway independently of PI3K. Tuberin-hamartin has also been shown previously to inhibit S6K1 activation upon the re-addition of amino acids to nutrient-starved cells (10–12) and to be associ-
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ated with mTOR (10). Collectively, the evidence supports the model that mTOR signaling is the target of TSC.

This work extends our current understanding of how TSC functions as a tumor suppressor within mammalian cells. We report that tuberin-hamartin blocks PKC/MAPK-mediated signaling to the downstream components of mTOR, 4E-BP1, and S6K1. It is known that both S6K1 and eEF2E4 function to drive cell growth and proliferation (20, 34). Given that tuberin is phosphorylated and inactivated by a PKC/MAPK signaling input, which is necessary for S6K1 activity and eEF2E4 function to drive cap-dependent translation, it is possible that the inappropriate inactivation of tuberin through constitutive PKC/ MAPK signaling may contribute to oncogenesis.

PI3K/Akt signaling inhibits the tumor suppressor function of tuberin-hamartin through the Akt-dependent phosphorylation of tuberin that occurs predominantly at Ser-939 and Thr-1462 (9, 10, 15, 32). Interestingly, phosphorylation of these sites also activates signaling to the downstream components of mTOR, 4E-BP1, and S6K1. It is known that both S6K1 and eIF4E function to drive cap-dependent translation, it is possible that the inactivation of tuberin-hamartin independently (Fig. 9), and phosphorylation of these overlapping RXRX(X/S/T) consensus sites within tuberin activates the tumor suppressor heterodimer to promote mTOR signaling. In support of our model, we show that the tuberin(S939A/T1462A) mutant, which cannot be phosphorylated by Akt (9), inhibits PKC/MAPK-mediated activation of S6K1 more potently than wild-type tuberin (Fig. 7A). Therefore, phosphorylation of these overlapping RXRX(X/S/T) consensus sites within tuberin by PI3K/Akt- and PKC/MAPK-mediated signaling are important for the regulation of the TSC1/2 proteins, tuberin and hamartin. It is probable that additional PKC/MAPK-mediated phosphorylation of tuberin contributes to the inhibition of the tuberin-hamartin heterodimer. One kinase candidate that may phosphorylate tuberin at these RXRX(X/S/T) consensus motifs was p90 ribosomal S6 kinase 1 (RSK1). However, overexpression of active RSK1 within HEK293E cells did not enhance the phosphorylation of tuberin (data not shown) implying that it is unlikely that RSK1 is the physiological kinase. Further studies will have to be carried out to identify the kinase(s) responsible for tuberin phosphorylation upon PKC/MAPK-mediated signaling. We are presently trying to determine which basophilic kinase phosphorylates the RXRX(X/S/T) consensus sites within tuberin upon activation of PKC/MAPK signaling. Some potential kinase candidates include conventional and novel PKCs and RSK2/3/4.

During the preparation of this manuscript, a number of studies characterized Rheb (Ras homologue enriched in brain) as a molecular target of TSC1/2 within flies and mammals (35–39). Drosophila genetics using epistasis analysis positioned Rheb downstream of dTSC1/2 and upstream of dTOR (35, 36). Research by Zhang et al. (37) identified dTSC2 as a Rheb GTPase-activating protein (GAP). Work from our laboratory demonstrated that Rheb specifically activated mTOR-mediated signaling toward S6K1 and 4E-BP1 that was blocked by tuberin and hamartin overexpression (39). Furthermore, mammalian studies show that tuberin is a RhebGAP (38, 39). Interestingly, the ability of tuberin to function as a RhebGAP is significantly enhanced when associated with its binding partner, hamartin (39). These studies reveal that Rheb is an upstream modulator of mTOR and a downstream target of TSC1/2 (see model, Fig. 9) that is conserved between flies and mammals.

How does the phosphorylation of tuberin regulate the tuberin-hamartin heterodimer? It is currently unclear whether Akt-dependent phosphorylation of tuberin disrupts the inter-

action of tuberin and hamartin due to conflicting data. Favoring Akt-mediated tuberin phosphorylation leading to dissociation of the tuberin-hamartin heterodimer, insulin stimulation, or Akt overexpression reduced the amount of hamartin that co-immunoprecipitated with tuberin in Drosophila-derived cell lines (6). Furthermore, a phosphomimetic tuberin protein (where the Akt-mediated phosphorylation sites were substituted to acidic residues) associated less favorably with hamartin (10). In contrast, two research groups (9, 32) showed that the Akt-dependent phosphorylation of tuberin within mammalian cells was not sufficient to disrupt the TSC tumor suppressor complex. Similarly, we did not observe any disruption of the tuberin-hamartin heterodimer upon PMA-induced tuberin phosphorylation (data not shown). It has also been reported that Akt-mediated phosphorylation of tuberin increases the turnover rate of both tuberin and hamartin (10, 32). We carried out 135Smethionine pulse-chase experiments in HEK293E cells overexpressing tuberin to address whether PI3K/Akt- or PKC/MAPK-mediated signaling could enhance the turnover rate of exogenous tuberin, and we saw that neither insulin nor PMA stimulation of HEK293E cells resulted in the significant loss of tuberin over an 8-h period (data not shown). This discrepancy may be a consequence of overexpression that may enhance the stability of tuberin. Many research groups (40–43) have also shown that 14-3-3 binds to the phosphorylated species of tuberin, and recently, p38MAPK-activated kinase MK2 was observed to phosphorylate tuberin on S1210 and direct 14-3-3 binding to tuberin (33). These studies provide an attractive mechanism where 14-3-3 association with tuberin can rapidly inhibit the tumor suppressor complex and presumably target tuberin for degradation. It is unlikely that MK2 is the candidate kinase that phosphorylates tuberin upon PKC/ MAPK signaling inputs as the p38MAPK/MK2 signaling pathway is not stimulated upon cell treatments with PMA (Fig. 8). Furthermore, stimulation of the p38MAPK signaling pathway does not result in the phosphorylation of tuberin at the consensus RXRX(X/S/T) phosphorylation sites (Fig. 8). Given that the basophilic kinases, MK2 and mitogen and stress-activated kinase (MSK)-1/2, are downstream of p38, these p38-regulated kinases are unlikely to mediate tuberin phosphorylation at these RXRX(X/S/T) sites.

Our data support the model that tuberin and hamartin function together as a heterodimer to inhibit mTOR signaling and reveal that tuberin is inhibited through both PI3K-dependent and -independent mechanisms. It is interesting to speculate that the tuberin-hamartin heterodimer functions as a pivotal sensor of PI3K/Akt and PKC/MAPK signaling pathways that, depending on the signal strength of either pathway, modulates the extent of mTOR signaling. Much is unknown regarding the mechanism by which tuberin-hamartin functions to inhibit mTOR, although recent research has shown that Rheb is a molecular target of TSC1/2 and an upstream regulator of mTOR. It will be interesting to see how this complex story unravels with further studies.

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