Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning

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Loss of tuberin, the product of TSC2 gene, increases mammalian target of rapamycin (mTOR) signaling, promoting cell growth and tumor development. However, in cells expressing tuberin, it is not known how repression of mTOR signaling is relieved to activate this pathway in response to growth factors and how hamartin participates in this process. We show that hamartin co-localizes with hypophosphorylated tuberin at the membrane, where tuberin exerts its GTPase-activating protein (GAP) activity to repress Rheb signaling. In response to growth signals, tuberin is phosphorylated by AKT and translocates to the cytosol, relieving Rheb repression. Phosphorylation of tuberin at serines 939 and 981 does not alter its intrinsic GAP activity toward Rheb but partitions tuberin to the cytosol, where it is bound by 14-3-3 proteins. Thus, tuberin bound by 14-3-3 in response to AKT phosphorylation is sequestered away from its membrane-bound activation partner (hamartin) and its target GTPase (Rheb) to relieve the growth inhibitory effects of this tumor suppressor.

Introduction

Tuberous sclerosis complex (TSC), caused by loss of function of either the TSC1 or -2 tumor suppressor genes, is an autosomal dominant disorder that leads to mental retardation, seizures, and the formation of tumors in various organs, including the brain, kidney, heart, and skin (Young and Povey, 1998; Gomez et al., 1999; Cheadle et al., 2000). The TSC1 gene encodes the 130-kD protein hamartin (van Slegtenhorst et al., 1997), and the TSC2 gene encodes the 198-kD protein tuberin (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). Hamartin contains two coiled-coil domains, which have been shown to mediate binding to tuberin (Hodges et al., 2001), forming a stable, functional tumor suppressor heterodimer within cells (Plank et al., 1998; van Slegtenhorst et al., 1998). Lesions that develop in TSC patients are histologically diverse; however, the tumors that arise as a result of loss of function of either TSC1 or -2 share common features, suggesting that hamartin and tuberin function within the same pathways to regulate cell cycle, cell growth, adhesion, and vesicular trafficking (van Slegtenhorst et al., 1998; Hengstschlager et al., 2001). Recent studies have indicated that the hamartin–tuberin heterodimer regulates cell growth and proliferation as a downstream component of the phosphoinositide 3-kinase (PI3K)–protein kinase B (PKB/AKT) signaling pathway, which modulates signal transduction through target of rapamycin (TOR) in both Drosophila melanogaster and mammalian cells (Manning and Cantley, 2003; Inoki et al., 2005). Several distinct yet complementary genetic and biochemical studies collectively show that tuberin is a GTPase-activating protein (GAP) for the small GTPase Ras homologue enriched in brain (Rheb), which activates TOR and its downstream targets, such as the ribosomal S6 kinase (RSK; Li et al., 2004a).

Although loss of tuberin promotes cell growth and tumorigenesis, cells expressing tuberin must also be able to relieve tuberin repression of mammalian TOR (mTOR) signaling during conditions of mitogenic sufficiency. In this regard, tuberin contains multiple sites for AKT, MAPK, RSK, and extracellular signal–regulated kinase phosphorylation (Dan et al., 2002; Liu et al., 2002; Manning et al., 2002; Li et al., 2003; Tee et al., 2003; Roux et al., 2004; Ballif et al., 2005). Although it is clear that activation of AKT blocks tuberin inhibition of TOR signaling (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), the mechanism by which
AKT inactivates this tumor suppressor is unknown (Bjornsti and Houghton, 2004). In addition, there are conflicting data regarding the subcellular localization of tuberin. For instance, independent studies report that tuberin can localize to the cytosol (Nellist et al., 1999), the membrane/particulate (100,000 g) fraction (Wienecke et al., 1995), and even the nucleus (Lou et al., 2001) of cells.

In this study, we sought to determine the mechanisms by which tuberin is regulated during cell growth. We found that tuberin is localized in membrane and cytosol fractions but not in nuclear fractions, and the translocation of tuberin from the membrane to cytosol is regulated by AKT signaling in response to growth factors. Phosphorylation of tuberin by AKT causes tuberin to become sequestered by 14-3-3 proteins in the cytosol. Mutation of two specific phosphorylation sites (S939 and S981) prevents the cytosolic translocation of tuberin from cellular membranes and results in a constitutively active protein that inhibits mTOR signaling. Importantly, tuberin phosphorylation by AKT does not affect its GAP activity toward Rheb in vitro but promotes Rheb-induced S6 kinase (S6K) 1 activation through increased Rheb-GTP loading in vivo. Therefore, it is likely that AKT phosphorylation inhibits tuberin as a result of 14-3-3 binding and cytosolic translocation rather than by impairing its catalytic GAP activity.

**Results**

**Tuberin localization is regulated by Ser/Thr phosphorylation in response to growth factor stimulation**

To investigate the subcellular localization of tuberin, mouse fibroblast (NIH3T3, Swiss3T3), rat kidney epithelial (TRKE2), human embryonic kidney (HEK293), and human breast cancer (MCF7) cell lines were fractionated, revealing that tuberin was detected within both the cytosolic and membrane fractions but not within the nuclear fraction (Fig. 1 A). Interestingly, tuberin in the membrane fraction migrated faster than tuberin within the cytosolic fraction (Fig. 1 A).

To determine whether this mobility shift was due to changes in phosphorylation, we treated these subcellular fractions with either a Ser/Thr or Tyr phosphatase (calf intestinal alkaline phosphatase [CIAP] or YOP protein tyrosine phosphatase, respectively). After CIAP treatment, the mobility of tuberin in the cytosol increased, resolving as a faster migrating band similar to tuberin purified from membrane fractions (Fig. 1 B). However, YOP did not affect tuberin mobility, indicating that the mobility shift was primarily due to phosphorylation of Ser/Thr residues. Treatment with CIAP resulted in both membrane and cytosolic tuberin migrating faster, indicating that tuberin within...
the cytosolic fraction is hyperphosphorylated. In addition, the serum-induced decrease in mobility of tuberin in both membrane and cytosolic fractions is likely driven by multiple phosphorylation events, as CIAP treatment (removing all Ser/Thr phosphorylation) increased the mobility of tuberin in both fractions.

To determine whether growth factor stimulation could also alter tuberin phosphorylation and subcellular localization, Swiss3T3 or MCF7 cells were serum starved and then stimulated with serum or insulin-like growth factor-1 (IGF-1) before fractionation (Fig. 1, C and D; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1), as were NIH3T3 (Fig. S1 A) and TRKE2 cells (Fig. S1 B). Like serum, IGF-1 increased the amount of tuberin in the cytosolic fraction relative to starvation conditions (Fig. 1, C and D). In serum-stimulated cells, phosphorylation of membrane-localized tuberin was also increased after 1 h (comparable to cytosolic tuberin in starved cells but less than cytosolic tuberin from serum-stimulated cells; Fig. 1 C), suggesting that phosphorylation at specific residues, rather than total levels of phosphorylation, was determining localization. By 6 h, tuberin became predominantly membrane localized, which correlated with a decrease in AKT activation (Fig. 1 C). Translocation of tuberin from the membrane to the cytosol was blocked by the PI3K inhibitors wortmannin and LY294002 (Fig. 1 D and Fig. S1 B), implicating PI3K signaling in tuberin localization to the cytosol.

**Tuberin translocation is mediated by AKT phosphorylation**

To determine whether AKT directed tuberin’s subcellular localization, tuberin from membrane and cytosolic compartments of NIH3T3 cells treated with IGF-1 or EGF was immunoprecipitated and detected with a (S/T) phosphosubstrate antibody that recognizes the consensus phosphorylation site for AKT and RSK containing phospho-Ser/Thr with Arg at position −5 and −3 (RXRXXpS/T) (Alessi et al., 1996; Obata et al., 2000; Yaffe et al., 2001; Roux et al., 2004). With equal tuberin loading (Fig. 2, A and B), the (S/T) phosphosubstrate antibody predominantly recognized tuberin within the cytosolic fraction, and the PI3K inhibitor wortmannin significantly reduced recognition of phosphorylated tuberin, suggesting that cytosolic tuberin was phosphorylated by AKT (Fig. 2, A and B). To confirm that activation of AKT directed tuberin to the cytosol, MCF7 cells stably transfected with a constitutively active AKT (myr-AKT) were examined and found to contain more cytosolic tuberin relative to wild-type MCF7 cells (Fig. 2 C). Recently, RSK was shown to phosphorylate tuberin at S1798 (Roux et al., 2004). To determine whether RSK phosphorylation of tuberin could regulate its localization, we generated a COOH-terminal deletion mutant of TSC2 at residue 1734 (referred to as Δ73) that lacks S1798 (Roux et al., 2004). Δ73 and wild-type tuberin had a similar distribution within membrane and cytosolic fractions under normal growth conditions (Fig. S1 C) and in response to serum (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1). In addition, overexpression of RSK1 did not affect localization of wild-type tuberin (Fig. S2 B). Collectively, these data indicate that tuberin residing in the cytosol is phosphorylated by AKT, suggesting that AKT (but not RSK) directly controls tuberin’s localization and possibly its activity.

**Mutation of AKT phosphorylation sites in tuberin alters its localization**

Tuberin contains multiple S/T phosphorylation sites (Fig. 3 A). Among these, S1254 has been shown to be phosphorylated by MK2 (Li et al., 2003), whereas S939 (Inoki et al., 2002;
Manning et al., 2002), S981 (Dan et al., 2002), S1130/S1132 (Inoki et al., 2002), and T1462 (Inoki et al., 2002; Manning et al., 2002) have been reported to be AKT phosphorylation sites in vivo and/or in vitro, and several of these also have the potential for binding to 14-3-3 (http://scansite.mit.edu; Yaffe et al., 2001). S981 is of particular interest, as it lies within the alternatively spliced exon 25 of tuberin. To examine these as candidate sites for the regulation of tuberin localization, we mutated these residues to alanine to create TSC2 constructs in frame with an NH2-terminal Flag epitope (Flag-TSC2): S939A, S981A, and 2A (S939A + S981A); T1462A and SATA (S939A + T1462A; Manning et al., 2002); and S1254A and S1130A/S1132A double mutant.

Wild-type and mutant TSC2 constructs were transfected into HEK293 cells and subjected to subcellular fractionation. As shown in Fig. 3 (B and C), S939A, S981A, and 2A mutants predominately localized to the membrane, as did the double alanine SATA mutant that lacks the S939 site. However, the T1462A single mutant partitioned in the cell similarly to wild-type tuberin (Fig. 3 B), indicating that T1462 phosphorylation was not directing translocation of tuberin from the membrane to the cytosol. These data were confirmed with a phosphospecific T1462 antibody, which recognized tuberin in both the membrane and cytosolic fractions equally (unpublished data). Furthermore, phosphorylation at both S939 and S981 contributes to cytosolic localization, as phosphorylation at S939 (determined with a phospho-S939 specific antibody) of the S981A mutant was not sufficient to partition tuberin to the cytosol (Fig. S2 C). Other tuberin mutants, S1254A, S1130A/S1132A (Fig. 3 C), ∆73, or S1338A (Fig. S1 C), also distributed equally between the membrane and cytosolic fractions. Thus, S939 and S981 phosphorylation are critical determinants of membrane versus cytosolic localization of tuberin.

14-3-3 proteins mediate translocation of tuberin into the cytosol

14-3-3 has been previously reported to directly interact with phosphorylated tuberin (Li et al., 2002; Nellist et al., 2003). S939 and S981 are predicted AKT phosphorylation and 14-3-3 interaction sites. When phosphorylated and nonphosphorylated S939 and S981 peptides were used in competition assays to block GST–14-3-3 interaction with tuberin, as shown in Fig. 4 A and Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1), phosphorylated but not nonphosphorylated S939 and S981 peptides clearly competed for the interaction of tuberin with several 14-3-3 isoforms. In addition, the amount of 2A mutant tuberin affinity purified by 14-3-3 was dramatically reduced relative to wild-type tuberin (Fig. 4 B). Importantly, inhibition of PI3K signaling by wortmannin ablated this 14-3-3 tuberin interaction (Fig. 4 B). These data indicate that S939 and S981 are critical sites of interaction between tuberin and 14-3-3 proteins and that this interaction is mediated by PI3K/AKT phosphorylation.

To demonstrate that tuberin binding to 14-3-3 was directly responsible for its translocation to the cytosol, we transfected HEK293 cells with the EGFP-R18 construct that expresses a peptide that disrupts 14-3-3 binding (Jin et al., 2004). As shown in Fig. 4 C, the R18 14-3-3 decoy clearly repressed cytosolic localization of tuberin, establishing a direct link between 14-3-3 and translocation of tuberin to the cytosol.

Hamartin enhances tuberin retention at the membrane

Hamartin possesses a predicted transmembrane domain and two coiled-coil domains that mediate its association with tuberin (van Slegtenhorst et al., 1997, 1998). We found that in both human and mouse cells, hamartin was only detected in the membrane fraction (Fig. 5, A and B; Fig. 3 B; unpublished data). To confirm that tuberin mutants that did or did not constitutively localize to the membrane retained their ability to bind hamartin, immunoprecipitation experiments were performed. Immunoprecipitation showed that, similar to wild-type tuberin, tuberin S939A, S981A, S1338A, ∆73, and 2A mutants retained their ability to interact with hamartin (Fig. S4, A, B, and C, available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1). To determine whether hamartin played a role in the subcellular localization of tuberin, we transfected wild-type, S939A,
and S981A Flag-TSC2 constructs into HEK293 cells with or without Myc- or Flag-TSC1. Although S939A and S981A mutants were primarily membrane localized, coexpression of hamartin increased membrane retention of both mutant and wild-type tuberin in a dose-dependent manner (Fig. 5, A and B). These data suggest that the interaction of hamartin with tuberin facilitates its localization to the membrane, implying that the tuberin–hamartin heterodimer functions in this subcellular compartment.

Importantly, only tuberin in the membrane fraction remained associated with hamartin: no hamartin could be co-immunoprecipitated with tuberin in the cytosolic fraction, indicating that translocation to the cytosol dissociated tuberin from hamartin (Fig. 5 C). Similarly, when Flag-TSC2 and Myc-TSC1 were cotransfected into MCF7 cells, tuberin and hamartin were observed by confocal microscopy to colocalize in a discrete, punctate pattern (Fig. S4 D). Cells that expressed wild-type Flag-TSC2 in the absence of Myc-TSC1 exhibited a more diffuse staining pattern than cells that overexpressed both Myc-TSC1 and Flag-TSC2 (Fig. S4 D, comparing γ and γ′ with β and β′). However, the 2A mutant, which was constitutively membrane localized as shown by cell fractionation, retained this punctate localization pattern even in the absence of exogenously expressed hamartin (Fig. S4 E).

**Colocalization of tuberin and Rheb is disrupted in response to growth factor stimulation**

Tuberin’s GAP target Rheb is farnesylated and predicted to be membrane localized (Clark et al., 1997), and recent data indicate...
that Rheb is localized in endomembranes (Takahashi et al., 2005). To determine whether phosphorylation at S939/S981 affected tuberin’s intrinsic GAP activity for Rheb, we first determined Rheb’s subcellular localization. Rheb was detected only in the membrane fractions from HEK293 (Fig. 3 B) and MCF7 (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1) as well as NIH3T3, Swiss3T3, and TRKE2 cells (not depicted). Treatment with EGF or wortmannin did not affect this localization over a period of 10 min to 3 h (Fig. S5 A and not depicted). Recognition of the 21-kD band in the membrane fraction using this anti-Rheb antibody was specific for Rheb, as antibody binding was ablated when Rheb RNAi was used to knock down Rheb (Fig. S5 B). When untagged TSC1/Flag-TSC2 and Myc-Rheb were cotransfected into HeLa cells, tuberin and Rheb were observed with confocal microscopy to colocalize in a discrete, punctate pattern in the absence of serum (Fig. 6). In contrast, stimulation with IGF-1 resulted in partitioning of wild-type tuberin away from Rheb (Fig. S5 B). When untagged TSC1/Flag-TSC2 and Myc-Rheb were cotransfected into HeLa cells, tuberin and Rheb were observed with confocal microscopy to colocalize in a discrete, punctate pattern in the absence of serum (Fig. 6).

In cells transfected with HA-tagged S6K, cotransfection of wild-type TSC1 and -2 expression constructs diminished phosphorylation of exogenously expressed S6K (Fig. 7 A). However, cotransfection of the 2A mutant with TSC1 resulted in an even more dramatic reduction in phospho-S6K levels (Fig. 7 A). Similar experiments were performed to determine the effect of S939A and S981A single and double mutants on phosphorylation of endogenous S6K. In the presence of serum, wild-type Flag-tuberin was located in the membrane and cytosol, whereas S939A, S981A, and 2A Flag-tuberin mutants remained primarily at the membrane (Fig. 7 B, top). AKT was equally activated by serum in cells expressing wild-type or mutant tuberin constructs, and endogenous hamartin remained membrane localized (Fig. 7 B). In cells transfected with wild-type tuberin, phosphorylation of endogenous S6K at T389 increased in response to serum, coincident with translocation of tuberin to the cytosol. However, in cells transfected with S939A, S981A, or 2A tuberin mutants, phosphorylation of S6K was inhibited, with the 2A mutant most effectively blocking S6K activation (Fig. 7 B). Thus, tuberin mutants that were retained at the membrane were constitutively active and exhibited enhanced ability to repress S6K activation. These data suggest that AKT phosphorylation of tuberin regulates its activity by decreasing the amount of tuberin located at the membrane, thereby reducing its inhibitory effect on the Rheb–mTOR–S6K signaling pathway.

Phosphorylation sites that determine localization and function of tuberin do not change its intrinsic RhebGAP activity in vitro

Physical sequestration of tuberin from its target Rheb suggested a mechanism whereby AKT modulated tuberin by partitioning it away from the membrane rather than altering its intrinsic GAP activity for Rheb. We compared the relative RhebGAP activity of wild-type TSC2 and 2A mutant (S939A and S981A) in cells during activation of the PI3K–AKT pathway (Fig. 8).

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**Figure 6.** Tuberin colocalization with Rheb is disrupted in response to growth factor stimulation. HeLa cells were transfected in serum-free media with Flag-TSC2 WT and Myc-Rheb along with an untagged TSC1 construct. After 12 h without serum, cells were treated with 30 ng/ml IGF-1 for 1 h. Localization of exogenous Flag-tuberin (green) and Myc-Rheb (red) was detected by double immunofluorescence labeling and confocal microscopy. Colocalization is detected in the merged image with the yellow signal (right).
To do this, we quantified the ratio of GTP and GDP Myc-Rheb (percentage of GTP bound Myc-Rheb) when these TSC2 constructs were coexpressed during a time course of insulin stimulation. The 2A mutant enhanced the GTPase function of Rheb more effectively than wild-type tuberin, as indicated by impaired accumulation of the active GTP form of Rheb after 15 min of insulin stimulation (32% GTP bound) when compared with wild-type TSC2 (42% GTP bound; Fig. 8 A). As an increase in GTP bound Rheb would be predicted to enhance mTOR-mediated cell signaling, we measured the activity of HA-S6K1 in these cells (Fig. 8 B). As expected, insulin-induced activation of S6K1 was markedly impaired in cells expressing the 2A mutant with the 2A mutant blocking insulin-induced activation of S6K1 by 50% (after 15 min) when compared with wild-type TSC2 (Fig. 8 B), reflecting the reduced levels of active GTP bound Rheb (Fig. 8 A).

To determine whether phosphorylation at S939 and S981 had an impact on tuberin’s intrinsic GAP activity, we analyzed the ability of wild-type or mutant tuberin proteins to activate Rheb GTPase in vitro. RhebGAP assays were conducted on immunoprecipitated tuberin–hamartin heterodimers containing either the wild-type or AKT-phosphorylation mutants of TSC2 (Fig. 9).

Figure 7. AKT-mediated phosphorylation of tuberin relieves repression of mTOR-S6K signaling. [A] HEK293 cells were transfected with HA-S6K, Myc-TSC1, and Flag-TSC2 constructs as indicated. Cells were serum starved for 24 h and treated with or without 30 ng/ml IGF-1 for 1 h. Cell lysates were immunoblotted with the indicated antibodies, and phospho-S6K levels were compared with the amount of HA-S6K protein levels. Phospho-AKT [S473] was blotted as a control for the effectiveness of IGF-1 treatment. [B] Western analyses of membrane and cytosolic fractions from HEK293 cells transfected with wild-type or mutant Flag-TSC2 constructs. After 9 h, serum was either removed or allowed to remain for 24 h. The protein levels of phosphorylated endogenous S6K were compared with total S6K protein levels. Phospho-AKT [S473] and phospho-AKT [T308] were blotted as controls for the effectiveness of the indicated treatments.

Figure 8. AKT phosphorylation of tuberin promotes Rheb-induced S6K1 activation through increased Rheb-GTP loading. (A) HEK293 cells coexpressing HA-S6K1, Myc-Rheb, Flag-TSC1, and either Flag-TSC2-WT or Flag-TSC2-2A (S939A and S981A) were serum starved and, where indicated, the PI3K–AKT signaling pathway was stimulated with 100 nM insulin for 7.5 and 15 min. These cells were subjected to in vivo radiolabeling, and the level of guanine nucleotide bound to immunoprecipitated Myc-Rheb was quantified. A representative blot from three independent biological replications is shown. The mean of the percentage of total Myc-Rheb bound to GTP (active state) is shown in the bar figure. (B) In parallel, HEK293 cells treated as in A were subjected to S6K1 activity assays. Protein levels of Flag-TSC1, Flag-TSC2, and AKT, and level of AKT phosphorylation at S473, were determined using Western analyses.
Tuberin–hamartin complexes containing wild-type tuberin from both unstimulated and insulin-stimulated cells enhanced the intrinsic GTPase activity of Rheb at comparable rates. Induction of tuberin phosphorylation by insulin was confirmed in these lysates by detection of phospho-T1462 (Fig. 9, bottom).

Furthermore, tuberin mutants lacking these sites (S939A, S981A, and 2A) also possessed similar RhebGAP activity to wild-type tuberin in vitro, even after insulin treatment (Fig. 9). These findings imply that AKT-mediated phosphorylation of tuberin does not directly alter the rates at which tuberin enhances the GTPase activity of Rheb, at least in vitro. However, these data support a mechanism whereby AKT phosphorylation of tuberin does not directly alter the rates at which tuberin enhances the GTPase activity of Rheb, at least in vitro. However, these data support a mechanism whereby AKT phosphorylation of tuberin does not directly alter the rates at which tuberin enhances the GTPase activity of Rheb, at least in vitro. However, these data support a mechanism whereby AKT phosphorylation of tuberin does not directly alter the rates at which tuberin enhances the GTPase activity of Rheb, at least in vitro. However, these data support a mechanism whereby AKT phosphorylation of tuberin does not directly alter the rates at which tuberin enhances the GTPase activity of Rheb, at least in vitro.

**A mechanism by which tuberin function is regulated through subcellular localization**

Based on our data, we propose a model where in the absence of growth stimulatory signals, hamartin facilitates localization of hypophosphorylated tuberin to membranes in physical proximity to Rheb. The membrane-associated tuberin–hamartin complex binds Rheb and acts as a GAP to inactivate membrane

Rheb signaling by stimulating GTP hydrolysis (Fig. 10 A). However, during mitogenic sufficiency (i.e., after growth factor stimulation), activation of PI3K signaling leads to activation of AKT, which then directly phosphorylates membrane-associated tuberin. In response to AKT phosphorylation, 14-3-3 proteins bind tuberin and sequester it in the cytosol. The deficiency of membrane-associated tuberin results in the accumulation of GTP bound Rheb, which leads to increased mTOR signaling, enhanced cell growth, and proliferation (Fig. 10 B).

**Discussion**

In this study, we provide evidence that AKT inhibits the tumor suppressor function of tuberin by altering its subcellular localization. Previously, AKT was shown to directly phosphorylate and inhibit tuberin function upon stimulation with growth factors (Marygold and Leevers, 2002). However, it was not known how AKT phosphorylation of tuberin regulated its function or how hamartin contributed to tuberin regulation of Rheb (Garami et al., 2003; Tee et al., 2003; Zhang et al., 2003). We have shown that although tuberin is found in both the membrane and cytosolic fractions of cells, cytosolic tuberin accumulates upon growth factor stimulation and is hyperphosphorylated. The translocation of tuberin from the membrane to the cytosol can be blocked by PI3K inhibitors, and AKT-phosphorylated tuberin is predominantly found in cytosolic fractions. The AKT phosphorylation sites S939 and S981 are...
crucial residues that affect tuberin localization, with localization of tuberin to the membrane accounting for the ability of tuberin to inhibit mTOR signaling via its GAP activity for Rheb.

We show that two tuberin phosphorylation sites, S939 and S981, are responsible for interaction with 14-3-3 in a phosphorylation-dependent manner. 14-3-3 proteins are highly acidic dimeric intracellular proteins that chiefly bind to phosphoserine motifs (Yaffe and Elia, 2001). They play a key regulatory role in many cellular processes, including signal transduction, apoptosis, and cell cycle checkpoint control (Muslin and Xing, 2000; Tzivion et al., 2001; Yaffe, 2002; Hermeking, 2003). In many instances, 14-3-3 binding leads to altered subcellular localization of target proteins, which modulates their function. As this appears to be the case for tuberin as well, we propose a mechanism whereby growth factor stimulation activates AKT, leading to tuberin phosphorylation and mislocalization within the cell. Although some studies have suggested that the tuberin–hamartin heterodimer is destabilized upon AKT-mediated phosphorylation of tuberin (Inoki et al., 2002; Potter et al., 2002), our data and those of others indicate that AKT-mediated phosphorylation of tuberin does not change its affinity for hamartin (Dan et al., 2002; Manning et al., 2002; Tee et al., 2003). An alternative hypothesis is that in response to phosphorylation of S939 and S981 by AKT, 14-3-3 binds to these phosphorylated motifs to localize tuberin in the cytosol, physically sequestering tuberin away from hamartin. The presence of two adjacent 14-3-3 binding sites at S939 and S981 of tuberin may be important, as they would be predicted to stabilize binding of tuberin in the central channel of the 14-3-3 dimer more effectively than would a single binding site.

In this regard, it is interesting that loss of either the S939 or S981 14-3-3 binding sites reduces localization of tuberin to the cytosol. As one of these binding sites is in the alternatively spliced exon 25 of tuberin, it suggests that alternative splicing may allow for wider or more varied regulation of this tumor suppressor. In splice isoforms lacking exon 25, the absence of the S981 binding site could result in retention at the membrane, potentially enhancing tuberin activity. This could provide a mechanism via alternative exon splicing for cells and tissues to regulate tuberin localization and function. It might be noted that in previous studies that failed to identify the S939 site within tuberin as a 14-3-3 binding site, the TSC2 construct used lacked the alternatively spliced exon 25 and the S981 14-3-3 binding site (Li et al., 2002).

Hamartin has been shown to significantly enhance tuberin’s GAP activity toward Rhyb GTPase (Garami et al., 2002), and our observations also emphasize the importance of the interaction between tuberin and hamartin as a functional unit. We found that hamartin directly interacts with tuberin and promotes tuberin colocalization at the membrane in proximity to its GAP target Rhyb and enhances tuberin’s ability to repress mTOR signaling. Both endogenously and exogenously expressed hamartin were detected in the membrane fraction of cells, even when cells were treated with serum or growth factors, data that are consistent with the previous reports that hamartin is a membrane protein (van Slegtenhorst et al., 1997; Plank et al., 1998). The colocalization of tuberin in proximity to its activation partner hamartin and its GAP target Rhyb at the membrane could explain why increased retention of tuberin at the membrane enhances its ability to inhibit phosphorylation of S6K. Given that the S939 and S981 residues lie distal from the COOH-terminal GAP region (residues 1517–1674) of tuberin and mutation of these sites does not affect the Rhyb GAP activity of tuberin, we conclude that phosphorylation of S939 and S981 does not directly regulate the GAP function of TSC2.

However, mutation of these sites significantly inhibits Rhyb-induced S6K1 activation through decreased Rhyb-GTP loading, suggesting that AKT regulates tuberin function by causing tuberin translocation to the cytosol, rather than by directly inhibiting its intrinsic GAP activity toward Rhyb.

**Materials and methods**

**Antibodies and reagents**

The following antibodies were used: tuberin, hamartin, lamin A/C, HA, and Myc (Santa Cruz Biotechnology, Inc.); Rhyb, phospho-tuberin (T1462), S6K, phospho-S6K (T389), AKT, phospho-AKT (S473), phospho-AKT (T308), and phospho-(S/T) AKT substrate (Cell Signaling Technology); Flag M2 and Flag M2 immobilized agarose beads (Sigma-Aldrich); LDH (Chemicon International); EGFP (Abcam); and β1-integrin (CLONTECH Laboratories, Inc.). The following reagents were used: EGF, insulin, and wortmannin (Sigma-Aldrich) and IGF-1 [R & D Systems]. Tuberin peptides were synthesized by W.M. Keck Biotechnology Resource Center.

**Constructs**

Full-length human TSC1 and -2 cDNAs (supplied by J. DeClue, National Cancer Institute, Bethesda, MD) were subcloned into pcDNA3.1 (Invitrogen) and pcCMV-Tag2 (Stratagene) expression vectors with NH2-terminal Myc or Flag epitopes, respectively. COOH-terminal truncation mutant of TSC2 (Δ73) [residues 1–1734] was created by EcoR V digestion followed by religation. TSC2 mutations were generated by site-directed mutagenesis (Stratagene).

Other constructs used in this study were generously provided as follows: Flag-TSC1 and HA-S6K from J. Blenis (Harvard Medical School, Boston, MA), HA-RSK1 from J. Avruch (Massachusetts General Hospital, Boston, MA), GFP-R18 from T. Pawson (Mount Sinai Hospital, Toronto, Canada), and the Flag-TSC2 S142A and T1462A from B. Manning (Harvard School of Public Health, Boston, MA). GST–14-3-3 constructs were described previously (Liu et al., 2002).

**Cell culture, transfection, and immunoprecipitation**

Cell lines were grown as follows: MCF7 cells were grown in improved minimum essential medium (BioSource International). TR2E2 cells were grown in DF8 complete medium (Walker and Ginsler, 1992). Hela cells were grown in MEM, and HEK293, Swiss3T3, and NIH3T3 cells were grown in DME (Life Technologies, Inc.). All media contained 10% FBS (Hyclone) unless otherwise noted. Myc-Akt MCF7 cells were cultured as previously described (DeGraffenried et al., 2003). Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to manufacturer’s instructions. Cells were lysed using PBS containing 0.1% SDS, 1% NP-40, 0.5% deoxycholic acid, 1 μM PMSF, 20 μg/ml aprotinin, 10 μM leupeptin, and 1 μM Na3VO4. For immunoprecipitation, cell lysates were immunoprecipitated with the indicated antibodies and protein A- or protein G-Sepharose beads (GE Healthcare) and washed with buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 1% Triton X-100, 100 mM NaCl, 50 mM Naf, 2 mM EDTA, 1 mM PMSF, and Complete protease inhibitor cocktail [Roche]). Immunocomplexes were subjected to SDS-PAGE and Western blotting.

**Subcellular fractionation and protein phosphatase treatments**

Cells (70–80% confluent in 15-cm plates) were washed and collected by scraping into ice-cold PBS, pelleted by centrifugation at 4°C, resuspended in hypotonic buffer (10 mM Hepes, pH 7.2, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 20 mM NaF, and 100 μM Na3VO4), and disrupted using a Dounce homogenizer. Crude nuclei and unbroken cells were then pelleted by centrifugation at 3,000 rpm at 4°C for 5 min. The postnuclear supernatant was separated by ultracentrifugation at 10,000 g for 1 h at 4°C. The supernatant, or cytosolic fraction, was removed and the pellet
was lysed in 1× lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na$_3$PO$_4$, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, and 1 μg/ml leupeptin). The insoluble fractions were removed by centrifugation at 14,000 rpm for 10 min, and the supernatant was collected as the membrane fraction. Crude nuclei were resuspended with hypotonic buffer and homogenized using a Dounce homogenizer. After centrifugation at 4°C for 5 min, the pellet was further washed with wash buffer [10 mM Tris-HCl, pH 7.4, 0.1% NP-40, 0.05% sodium deoxycholate, 10 mM NaCl, and 3 mM MgCl$_2$] and lysed in high-salt lysis buffer (20 mM Heps, pH 7.4, 0.5 M NaCl, 0.5% NP-40, and 1.5 mM MgCl$_2$). All lysis and wash buffers contained 1× Complete protease inhibitor cocktail. The nuclear, membrane, and cytosolic lysates were normalized using the BCA Protein Assay kit (Pierce Chemical Co.) and equal amounts of membrane and cytosolic lysates were treated with 100 μM phospho-Scott$^{939}$ or Scott$^{981}$ tuberin peptide (SGGSFRARST[939]LNERPK) or phospho-Ser$^{939}$ or Ser$^{981}$ tuberin peptide (SGGGFRCRSS[981]VSEHV).

GST-14-3-3 pull-down assays

GST pull-down assays were performed as previously described (Liu et al., 2002). For peptide competition assays, incubations were performed in the presence or absence of 100 μM phospho-Scott$^{939}$ or Scott$^{981}$ tuberin peptide (SGGSFRARST[939]LNERPK) or phospho-Ser$^{939}$ or Ser$^{981}$ tuberin peptide (SGGGFRCRSS[981]VSEHV).

Rheb GAP and S6K1 assay

Anti-Flag M2 antibody was used to immunoprecipitate Flag-tuberin from HEK293E cells. Immunocomplexes of TSC1/TSC2 were used for in vitro kinase/Akt pathway regulates tuberous sclerosis tumor suppressors.

In vivo Rheb nucleotide binding

Analysis of Rheb guanine nucleotide binding in cells was determined as previously described (Lee et al., 2003). RhebGAP assay assayed as previously described (Lee et al., 2003). α[32P]GTP and α[32P]GDP were eluted from Rheb and resolved by thin layer chromatography on PEI cellulose (Sigma-Aldrich) with KH$_2$PO$_4$. The relative levels of radiolabeled GDP and GDP were quantified with a phosphorimager.

Intracellular localization of wild-type tuberin and 2A mutant and colocalization studies was determined by immunofluorescence analysis of HeLa cells. Transfected cells were plated onto glass chamber slides in the absence of serum. After 12 h of serum starvation, transfected cells were treated with either 20% FBS or 30 ng/ml IGF-1 for 1 h. Cells were then fixed in 5% formaldehyde plus 10% acetic acid for 1 h at 4°C, and nonspecific antigens were blocked for 1 h in PBS containing 7.5% BSA at 37°C. Rabbit anti-Flag (Sigma-Aldrich) and/or mouse anti-Myc (Santa Cruz Biotechnology, Inc.) primary antibodies were incubated in PBS/7.5% BSA at 37°C for 2 h. Primary antibodies were detected with FITC-conjugated goat anti-rabbit (Abcam) and Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories) antibodies, respectively.

Microscopy

Fluorescence images were analyzed either on a confocal microscope (Fluoview Scanning Laser Biological Microscope IX 70 system; Olympus) equipped with two lasers (Ar 488 and Kr-Ar 488–564) using UplanFl 40× objective. Photoshop 8.0 software (Adobe) was used for minor adjustments and processing of images.

Online supplemental material

Fig. S1 shows that AKT-mediated phosphorylation of tuberin leads to subcellular translocation. Fig. S2 shows that S939 and S981 are required for altered subcellular localization of tuberin. Fig. S3 demonstrates that 14-3-3 proteins bind phosphorylated S939 and S981 residues. Fig. S4 demonstrates that tuberin mutants retain their ability to interact with hamartin. Fig. S5 shows that Rheb is retained in the membrane fraction in the presence of growth factor treatment. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200507119/DC1.

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