

# Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway

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## Summary

The S/T-protein kinases activated by phosphoinositide 3-kinase (PI3K) regulate a myriad of cellular processes. Here, we show that an approach using a combination of biochemistry and bioinformatics can identify substrates of these kinases. This approach identifies the *tuberous sclerosis complex-2* gene product, tuberin, as a potential target of Akt/PKB. We demonstrate that, upon activation of PI3K, tuberin is phosphorylated on consensus recognition sites for PI3K-dependent S/T kinases. Moreover, Akt/PKB can phosphorylate tuberin *in vitro* and *in vivo*. We also show that S939 and T1462 of tuberin are PI3K-regulated phosphorylation sites and that T1462 is constitutively phosphorylated in PTEN<sup>-/-</sup> tumor-derived cell lines. Finally, we find that a tuberin mutant lacking the major PI3K-dependent phosphorylation sites can block the activation of S6K1, suggesting a means by which the PI3K-Akt pathway regulates S6K1 activity.

## Introduction

Class I phosphoinositide 3-kinases (PI3Ks) are activated by many extracellular growth and survival stimuli. These lipid kinases catalyze the production of the second messengers phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4P<sub>2</sub>) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5P<sub>3</sub>; reviewed by Katso et al., 2001; Rameh and Cantley, 1999). Downstream targets containing specialized domains, such as pleckstrin-homology (PH) domains, that specifically bind to these lipid products of PI3K are then activated. These activated proteins control a wide array of cellular processes, including survival, proliferation, protein synthesis, growth, metabolism, cytoskeletal rearrangements, and differentiation. However, there is still much we do not know about the signaling events leading from activation of PI3K effectors to downstream changes in cell physiology.

Serine/threonine (S/T) protein kinases can account for much of the functional diversity of PI3K signaling (reviewed by Toker, 2000; Vanhaesebroeck and Alessi, 2000). Akt/protein kinase B and the 70 kDa-S6 kinase 1 (S6K1) are the best characterized of the PI3K-regulated S/T kinases. The mitogen-stimulated activation of both

of these kinases is blocked by PI3K-specific inhibitors (Burgering and Coffey, 1995; Chung et al., 1994; Franke et al., 1995). Akt contains a PH domain that is specific to PtdIns-3,4P<sub>2</sub> and PtdIns-3,4,5P<sub>3</sub> (Franke et al., 1997). Akt is thereby recruited to these PI3K-generated second messengers and to the PDK1 protein kinase, which also specifically binds to these lipids (Stokoe et al., 1997). PDK1 then phosphorylates and activates Akt (Alessi et al., 1997).

The regulation of S6K1 is much more complex, with both PI3K-dependent and -independent signaling pathways involved in its activation (Chung et al., 1994; Weng et al., 1995). Several PI3K-regulated effectors are known to participate in the activation of S6K1 including PDK1, PKC $\zeta/\lambda$ , Cdc42, Rac1, and Akt (Burgering and Coffey, 1995; Chou and Blenis, 1996; Kohn et al., 1998; Pullen et al., 1998; Romanelli et al., 1999). However, the molecular mechanism of how these contribute to S6K1 activation remains unclear (reviewed by Martin and Blenis, 2002). In addition to mitogen-regulated signaling to S6K1, the metabolic state of the cell and the availability of nutrients control S6K1 activation through the mammalian target of rapamycin (mTOR, also known as FRAP, RAFT, and RAPT; Dennis et al., 2001; Hara et al., 1998). Recent studies suggest that mTOR is also regulated by mitogenic signals (Fang et al., 2001). Interestingly, it has been suggested that the point of convergence of the mitogenic and nutrient-sensing signals in the regulation of S6K1 may be at the level of Akt directly phosphorylating mTOR (Nave et al., 1999; Scott et al., 1998). However, this phosphorylation does not appear to affect mTOR activity or S6K1 activation (Sekulic et al., 2000). Thus, of the PI3K-regulated effectors thought to participate in S6K1 activation, the molecular basis of how Akt regulates S6K1 remains the least well understood.

Akt itself has been implicated in many of the PI3K-regulated cellular events, and several substrates have been shown to be phosphorylated *in vitro* and/or *in vivo* by Akt (recently reviewed by Brazil and Hemmings, 2001; Vanhaesebroeck and Alessi, 2000). Therefore, the total cellular effect of PI3K activation and subsequent activation of Akt is mediated through a variety of different targets. However, it seems unlikely that the large array of processes controlled by the PI3K-Akt pathway can be accounted for by our current knowledge of downstream targets.

Here, we have developed an approach to screen for substrates of PI3K-dependent S/T kinases, such as Akt. This approach uses phospho-specific antibodies generated against a phosphorylated protein kinase consensus recognition motif in combination with a protein database motif scanning program called Scansite (<http://scansite.mit.edu>; Yaffe et al., 2001). Scansite is a web-based program that searches protein databases for optimal substrates of specific protein kinases and for optimal binding motifs for specific protein domains with data generated by peptide library screens (e.g., Obata et al., 2000; Songyang and Cantley, 1998; Yaffe and Cantley, 2000; Yaffe et al., 2001). The phospho-motif antibody is used to recognize proteins phosphorylated

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Table 1. Scansite Predictions for Akt Substrates at Given Molecular Weight Ranges

MW Range <sup>a</sup>	Predicted MW	Predicted Best Substrates <sup>b</sup>	Position	Human Sequence <sup>c</sup>	Percentile <sup>d</sup>
42–48 kDa	42 kDa	FUCT-III	332	TLRPRSF <sup><b>S</b></sup> WALDFCK	0.003%
	43 kDa	FUCT-V	345	TLRPRSF <sup><b>S</b></sup> WALAFCK	0.007%
	47 kDa	<b>GSK-3<math>\beta</math></b>	9	SGRPR <sup><b>T</b></sup> TSFAESCKP	0.009%
48–52 kDa	50 kDa	TRF-1	273	SKRTR <sup><b>T</b></sup> ITSQDKPSG	0.019%
	51 kDa	<b>GSK-3<math>\alpha</math></b>	21	SGRART <sup><b>S</b></sup> SFAEPGGG	0.026%
	50 kDa	TIS11D	125	KFRDR <sup><b>S</b></sup> FSENGDRSQ	0.026%
175–205 kDa	201 kDa	<b>Tuberin</b>	939	SFRAR <sup><b>S</b></sup> STSLNERPKS	0.005%
	201 kDa	<b>Tuberin</b>	1462	GLRPR <sup><b>G</b></sup> Y <sup><b>T</b></sup> ISDSAPS	0.009%
	197 kDa	<b>CHD-1</b>	1096	RSRSRRYSGSDSDSI	0.013%

<sup>a</sup>The approximate molecular weight range for protein bands blotted by the Akt-pSub antibody in a PI3K-dependent manner (see Figure 1A).

<sup>b</sup>As predicted by the Scansite program (<http://scansite.mit.edu>; Yaffe et al., 2001), these are the highest scoring Akt substrate sites in all human proteins in the SWISS-PROT protein database in the given molecular weight range.

<sup>c</sup>The sequence flanking the predicted site of phosphorylation (shown in bold) is given.

<sup>d</sup>The percentile reflects the overall score of the predicted site relative to every other serine and threonine scored in the SWISS PROT database (see Yaffe et al., 2001).

determine that the proteins recognized by the Akt-pSub antibody at approximately 45 and 50 kDa are GSK3 $\beta$  and GSK3 $\alpha$ , respectively (data not shown). Importantly, the previously characterized PI3K-dependent phosphorylation sites on these proteins are serines (Cross et al., 1995), demonstrating that the antibody, raised against phosphothreonine peptides, recognizes both phosphoserines and phosphothreonines in the sequence context of RxRxxS/T.

Because of the success in correctly predicting known Akt substrates, we applied this approach to other PI3K-dependent bands on the Akt-pSub immunoblot. In order to identify a candidate for the protein band at approximately 180 kDa (marked with a double asterisk in Figure 1A), we used Scansite to search for potential Akt substrates in the 175–205 kDa range. The sites with the highest Akt substrate scores in this size range were two sites in the *tuberous sclerosis complex-2* gene product tuberin and another site in chromodomain-helicase-DNA binding protein-1 (CHD-1; Table 1). Interestingly, recent *Drosophila* genetic studies have suggested that tuberin might function in a pathway downstream or parallel to the *Drosophila* homologs of the insulin receptor, PI3K, and Akt (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001).

In order to determine if this 180 kDa PI3K-dependent phosphoprotein is mouse tuberin, we used an anti-tuberin antibody to immunoprecipitate endogenous tuberin. Tuberin from anti-tuberin but not control IgG immunoprecipitates is blotted by the Akt-pSub antibody in response to PDGF stimulation, and this blotting is inhibited by wortmannin (Figure 1B). The 180 kDa band recognized by the Akt-pSub antibody from cell lysates is greatly diminished in the supernatants of these immunoprecipitations, demonstrating that this band is tuberin (data not shown). To confirm that tuberin is phosphorylated downstream of PI3K signaling, we introduced exogenous flag-tagged human tuberin into NIH-3T3 cells. In anti-flag immunoprecipitates from flag-tuberin-expressing cells, tuberin is recognized by the Akt pSub antibody in a PDGF-dependent manner (Figure 2A, top). Two different PI3K-specific inhibitors, wortmannin and LY294002, inhibit the reactivity of this antibody with tuberin. However, rapamycin has no effect on the recogni-

tion of tuberin by the Akt-pSub antibody. Interestingly, rapamycin did inhibit Akt-pSub antibody blotting of a 28 kDa band (Figure 2A, bottom), which we have identified as ribosomal S6 (data not shown). This result is consistent with the RxRxxS motif on S6 that is known to be phosphorylated by S6K1 (Flotow and Thomas, 1992). Therefore, mammalian tuberin is phosphorylated by a PI3K-dependent S/T kinase on a site recognized by this RxRxxS/T-motif antibody.

We next determined the time course of growth factor-induced RxRxxS/T phosphorylation of tuberin. Anti-flag immunoprecipitates were isolated from serum-starved flag-tuberin-expressing NIH-3T3 cells exposed to PDGF for various durations. PDGF stimulates reactivity with the Akt-pSub antibody within 15 min, and the signal peaks by 30 to 60 min (Figure 2B, top). This correlates well with activation of Akt in these cells, as scored by phosphorylation of Akt on S473 (Figure 2B, bottom). The phosphorylation of tuberin at sites recognized by this antibody slowly diminishes over the course of 10 hr. This is a similar time course to that seen for dephosphorylation of the well-characterized Akt substrates GSK3 $\alpha$  and - $\beta$  following PDGF stimulation (Figure 2B, bottom).

Tuberin is known to form a complex with the *TSC1* gene product hamartin (Nellist et al., 1999). We find that phosphorylation of tuberin does not affect this association. Despite the large change in phosphorylation of tuberin, the amount of hamartin that coimmunoprecipitates with tuberin is constant throughout the time course (Figure 2B, third panel from top). Furthermore, tuberin analyzed from hamartin immunoprecipitations is also phosphorylated on sites recognized by the Akt-pSub antibody (data not shown).

The growth factor-stimulated and PI3K-dependent phosphorylation of tuberin is not limited to PDGF-treated NIH-3T3 cells. Flag-tuberin was expressed in both human embryonic kidney-293 (HEK-293) cells and the human osteosarcoma cell line U2OS. Tuberin from insulin-stimulated HEK293 cells (Figure 2C) or IGF1-stimulated U2OS cells (Figure 2D) is recognized on Akt-pSub immunoblots. Once again, the growth factor-induced recognition of tuberin by this antibody is greatly diminished by inhibition of PI3K with wortmannin. Therefore, activation of PI3K by different growth factors in a

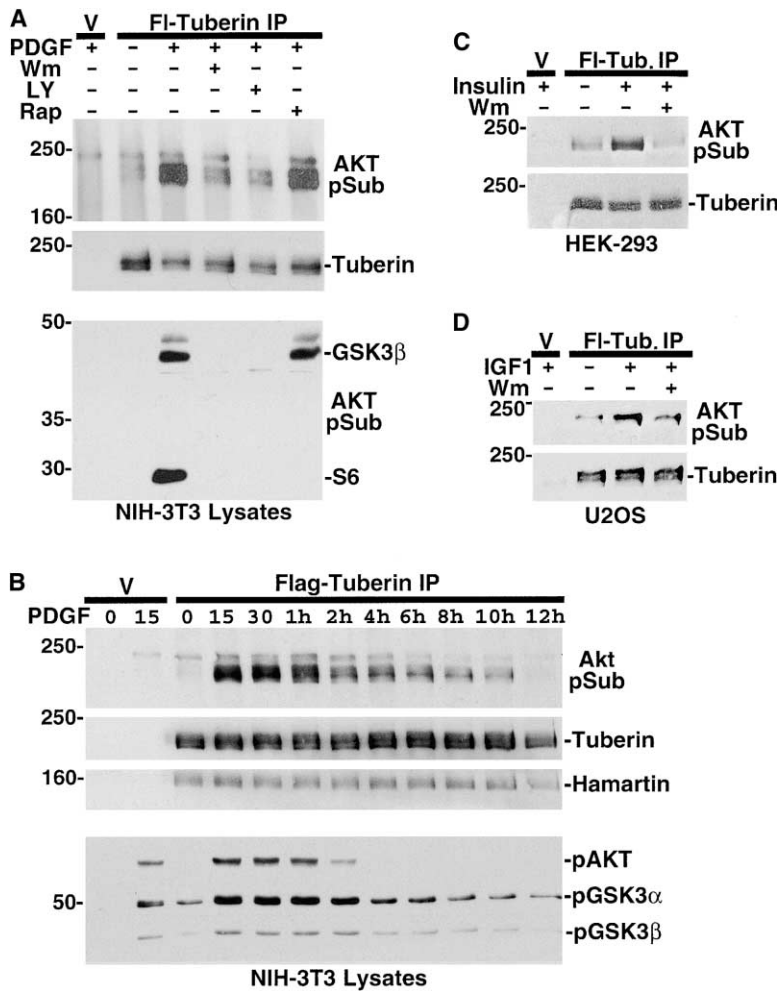


Figure 2. Tuberin Is Phosphorylated in a PI3K-Dependent Manner

(A) PI3K-dependent recognition of human tuberin by the Akt-pSub antibody. NIH-3T3 cells were transfected with FLAG-vector (V) or FLAG-tuberin constructs and were then serum starved. Cell lysates were prepared from cells left untreated, stimulated for 15 min with 10 ng/ml PDGF, or treated with 100 nM wortmannin (Wm), 10  $\mu$ M LY294002 (LY), or 25 nM rapamycin (Rap) for 15 min prior to stimulation with PDGF. Proteins were immunoprecipitated with M2 agarose and were immunoblotted with the Akt-pSub antibody (top), then, following stripping, were blotted with a tuberin antibody (middle). Proteins from total cell lysates were also blotted with the Akt-pSub antibody (bottom). The proteins identified in this molecular mass range are labeled. (B) Time course of PDGF-stimulated phosphorylation of tuberin on site(s) recognized by the Akt-pSub antibody. NIH-3T3 cells were transfected and serum starved as above. Cell lysates were prepared from cells stimulated with 10 ng/ml PDGF for the duration indicated (15 min to 12 hr). Proteins were then immunoprecipitated with M2 agarose and blotted with the Akt-pSub antibody (top) and the hamartin antibody (third panel from top). The region shown in the top panel was stripped and reprobed with the tuberin antibody (second panel from top). Proteins from total cell lysates were blotted with the Akt-pS473 antibody and the GSK-3 $\alpha$ / $\beta$ -pS21/S9 antibody (bottom).

(C) Tuberin is phosphorylated in response to insulin stimulation of HEK-293 cells in a PI3K-dependent manner. HEK-293 cells were transfected and serum starved as described in (A), and cell lysates were prepared from cells that were left untreated, stimulated for 15 min with 100 nM insulin, or treated with

100 nM wortmannin (Wm) prior to stimulation with insulin. Proteins immunoprecipitated with M2 agarose were blotted with the Akt-pSub antibody (top), followed by stripping and reprobing with the tuberin antibody (bottom).

(D) Tuberin is phosphorylated in response to IGF1 stimulation of U2OS cells in a PI3K-dependent manner. U2OS cells were transfected and treated as described in (A) and (C), but 10 ng/ml IGF1 was used instead of insulin.

variety of mammalian cell lines leads to phosphorylation of tuberin.

#### Akt Phosphorylates Tuberin In Vitro and In Vivo

Since residues S939 and T1462 of human tuberin were predicted by Scansite to be excellent sites for phosphorylation by Akt, we asked whether Akt could phosphorylate tuberin in vitro. Akt or kinase-dead Akt<sup>K179D</sup> (Akt-KD) was incubated with anti-flag immunoprecipitates from vector- or FLAG-tuberin-transfected wortmannin-treated cells. Autophosphorylation of Akt but not Akt-KD is detected in these kinase reactions (Figure 3A, top). Akt phosphorylates tuberin specifically from flag-tuberin immunoprecipitates, whereas no phosphate is incorporated into tuberin when exposed to Akt-KD (Figure 3A, top right). Therefore, the phosphorylation of tuberin in this experiment is specific to Akt kinase activity. As expected, this phosphorylation is on a site recognized by the Akt-pSub antibody (Figure 3A, second panel from top). Therefore, Akt can phosphorylate tuberin in vitro at a site, or sites, recognized by this RxRxxpS/T-motif antibody.

We next determined whether Akt could affect tuberin phosphorylation in vivo. Cotransfection of flag-tuberin and HA-Akt into HEK-293 cells leads to at least a 10-fold increase over vector-transfected cells in serum-stimulated phosphorylation of tuberin from flag-tuberin immunoprecipitates, as detected by Akt-pSub immunoblot (Figure 3B, second panel from top). As with phosphorylation of tuberin by endogenous kinase, this phosphorylation is greatly decreased by inhibition of PI3K (Figure 3B, top panel). Cotransfection of flag-tuberin with HA-Akt-KD did not lead to any increase in tuberin phosphorylation compared to vector alone. Once again, this phosphorylation event does not affect the amount of hamartin that coimmunoprecipitates with tuberin (Figure 3B, fourth panel from top). Interestingly, both HA-Akt and HA-Akt-KD are detected specifically in flag-tuberin but not control flag immunoprecipitates (Figure 3B, second panel from bottom). Reciprocally, flag-tuberin from these same lysates is detected specifically in HA-Akt but not control HA immunoprecipitates (Figure 3C), demonstrating that tuberin and Akt can physically associate. This association is not affected by treatment of

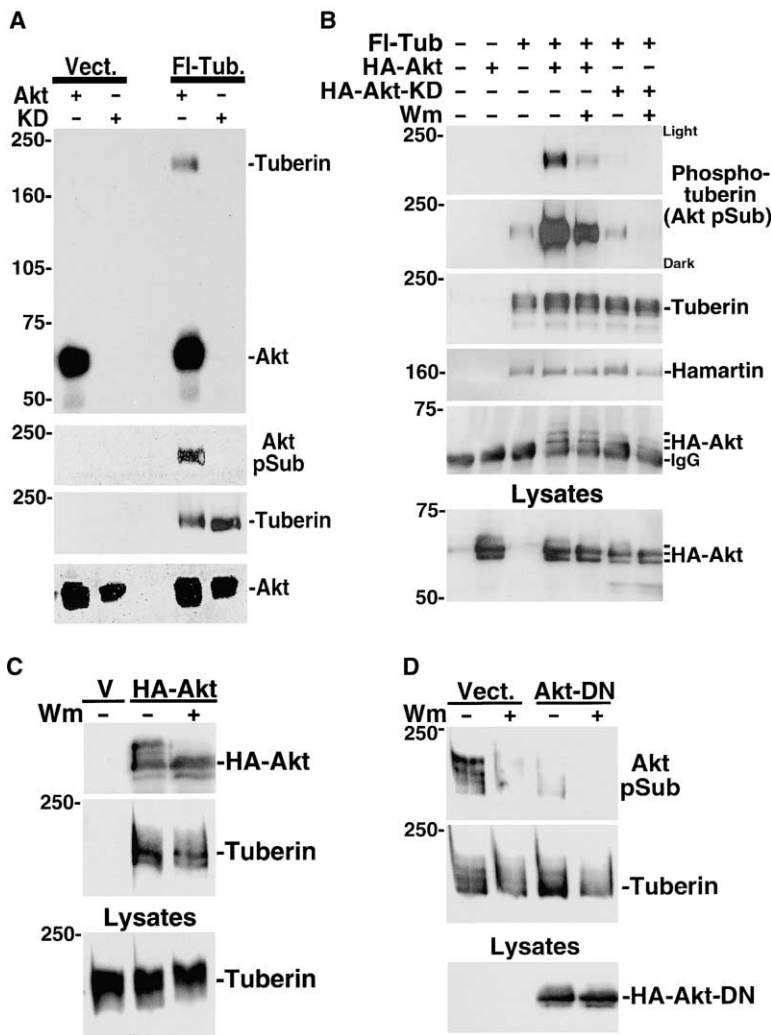


Figure 3. Akt Phosphorylates Tuberin on Sites Recognized by the Akt-pSub Antibody Both In Vitro and In Vivo

(A) Akt can phosphorylate tuberin in vitro. HEK-293 cells were transfected with HA-Akt, HA-Akt-KD, FLAG-vector, or FLAG-tuberin expression constructs. The corresponding proteins were isolated by immunoprecipitation, and in vitro kinase assays were performed (top; see Experimental Procedures). Proteins from these assays were also blotted with the Akt antibody (bottom), the Akt-pSub antibody (second panel from top), and, following stripping, with the tuberin antibody (third panel from top).

(B) In vivo effect of Akt or Akt-KD expression on recognition of tuberin by the Akt-pSub antibody. In HEK-293 cells, FLAG-vector or FLAG-tuberin constructs were cotransfected with either vector, HA-Akt, or HA-Akt-KD constructs. Cell lysates were prepared from cells in full serum or full serum with 100 nM wortmannin (Wm) for 15 min. Proteins immunoprecipitated with M2 agarose were analyzed by blotting with the Akt-pSub antibody (top two panels; light and dark exposures of same blot), the tuberin antibody (following stripping of the section shown in the top panels; third panel from top), the hamartin antibody (fourth panel from top), or the HA antibody (fifth panel from top). Proteins from the total cell lysates were blotted with the HA antibody (bottom).

(C) Coimmunoprecipitation of HA-Akt and FLAG-tuberin. HEK-293 cells were transfected and treated as in (B). Proteins from anti-HA immunoprecipitations were blotted with the HA antibody (top) and the tuberin antibody (middle). Proteins from total cell lysates were blotted with the tuberin antibody (bottom).

(D) A dominant-negative Akt blocks recognition of tuberin by the Akt-pSub antibody. In HEK-293 cells, HA-vector or HA-Akt-DN constructs were cotransfected with the FLAG-tuberin construct, and cell lysates were prepared as in (B). FLAG-tuberin was immunoprecipitated from these lysates and was blotted with the Akt-pSub antibody (top), then, following stripping, was blotted with the tuberin antibody (middle). Proteins from total cell lysates were blotted with the HA antibody (bottom).

tuberin construct, and cell lysates were prepared as in (B). FLAG-tuberin was immunoprecipitated from these lysates and was blotted with the Akt-pSub antibody (top), then, following stripping, was blotted with the tuberin antibody (middle). Proteins from total cell lysates were blotted with the HA antibody (bottom).

cells with wortmannin. We have been unable to detect an association between endogenous Akt and endogenous tuberin, perhaps due to the transient nature of the association and/or protein level limitations. In agreement with a previous report (van Weeren et al., 1998), we find that Akt-KD does not act as a dominant-negative to block phosphorylation of substrates by endogenous Akt. We therefore used a HA-Akt<sup>T308A/S473A</sup> mutant (Akt-DN), with its two activating phosphorylation sites mutated, as a dominant-negative Akt to test for blockage of tuberin phosphorylation. Compared to cells expressing vector alone, the cells expressing Akt-DN had greatly reduced tuberin phosphorylation (Figure 3D). Together with the in vitro phosphorylation of tuberin by Akt, these results are consistent with Akt being the PI3K-dependent S/T kinase that phosphorylates tuberin upon growth factor stimulation of mammalian cells.

#### S939 and T1462 Are the Primary PI3K-Dependent Phosphorylation Sites on Tuberin

As indicated above, two sites on human tuberin (S939 and T1462) score in the top 0.009 percentile as likely

Akt phosphorylation sites using Scansite. Four additional sites (S1130, S981, S1132, and S1798) also score as possible Akt sites. All of these sites are conserved in other vertebrate tuberins (mouse, rat, and *Takifugu*). Since this pathway appears to be conserved between mammals and *Drosophila* (Gao and Pan, 2001; Potter et al., 2001), we analyzed *Drosophila* tuberin by Scansite with the hope of identifying candidates for the physiologically relevant Akt site(s). Scansite predicts that *Drosophila* tuberin contains three potential Akt phosphorylation sites, and two of these sites, S924 and T1518, align quite well with S939 and T1462 in human tuberin (Figure 4A). We therefore tested whether these two sites were the primary sites of PI3K-dependent phosphorylation on tuberin.

In order to determine if S939 and T1462 were phosphorylated in response to PI3K activation, we immunoprecipitated flag-tagged wild-type tuberin, tuberin<sup>S939A</sup>, tuberin<sup>T1462A</sup>, and tuberin<sup>S939A/T1462A</sup> from HEK-293 cells and detected phospho-tuberin by Akt-pSub immunoblot. As shown above, under full serum conditions wild-type tuberin is recognized by the Akt-pSub antibody, and this

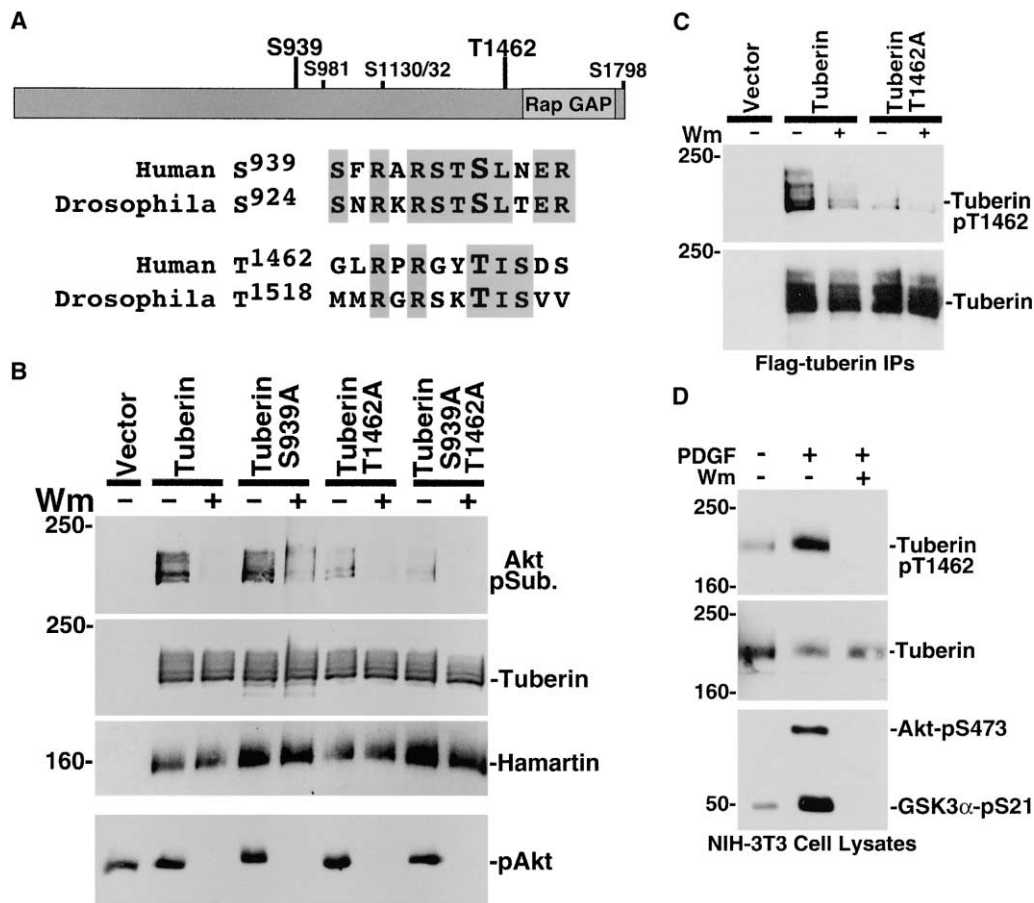


Figure 4. S939 and T1462 Are the Major PI3K-Dependent Phosphorylation Sites on Tuberin

(A) Predicted Akt phosphorylation sites on human tuberin and alignment with conserved sites on *Drosophila* tuberin. Shown are the six sites on human tuberin predicted by the Scansite program to be Akt phosphorylation sites. S939 and T1462 are given the highest score by Scansite and are shown aligned with the corresponding sites on *Drosophila* tuberin.

(B) Mutation of S939 and T1462 disrupts the recognition of tuberin by the Akt-pSub antibody. HEK-293 cells were transfected with FLAG-vector, FLAG-tuberin, FLAG-tuberin<sup>S939A</sup>, FLAG-tuberin<sup>T1462A</sup>, or FLAG-tuberin<sup>S939A/T1462A</sup>. Cell lysates were prepared from cells in full serum or full serum with 100 nM wortmannin (Wm) for 15 min. Proteins immunoprecipitated with M2 agarose were blotted with the Akt-pSub antibody (top), the tuberin antibody (following stripping of the section shown in the top panel; second panel from top), and the hamartin antibody (third panel from top). Proteins from total cell lysates were blotted with the Akt-pS473 antibody.

(C) Characterization of the tuberin-pT1462 antibody. HEK-293 cells were transfected and treated as in (B). Immunoprecipitated FLAG-tuberin was blotted with the tuberin-pT1462 antibody (top), then, following stripping, was blotted with the anti-tuberin antibody (bottom).

(D) Endogenous tuberin is phosphorylated on T1462 in response to PI3K activation. Cell lysates were prepared from serum-starved NIH-3T3 cells that were left untreated, stimulated for 15 min with 10 ng/ml PDGF, or treated with 100 nM wortmannin (Wm) for 15 min prior to stimulation with PDGF. Proteins were blotted with the tuberin-pT1462 antibody (top), the tuberin antibody (following stripping of the section shown in the top panel; middle), and both the Akt-pS473 and GSK-3 $\alpha$ -pS21 antibodies (bottom).

is inhibited by wortmannin treatment (Figure 4B, top). The tuberin<sup>S939A</sup> mutant is similarly blotted, but with a slight decrease in the intensity of the slowest migrating form, despite more tuberin<sup>S939A</sup> than wild-type tuberin being immunoprecipitated in this experiment (Figure 4B, second panel from top). There is a large decrease in blotting of the tuberin<sup>T1462A</sup> mutant (Figure 4B, top, compare lanes 2 and 6), and there is a further decrease in blotting of the tuberin<sup>S939A/T1462A</sup> double mutant (Figure 4B, top, compare lanes 6 and 8). The fact that the double mutant is still weakly recognized by the Akt-pSub antibody in a PI3K-dependent manner indicates that at least one of the other predicted Akt sites in human tuberin is likely to be phosphorylated in this mutant. However, these data demonstrate that S939 and T1462 are the

primary sites recognized by this antibody in response to activation of the PI3K-Akt pathway. Complex formation with hamartin is not affected by these phosphorylation-site mutations; the amount of hamartin that coimmunoprecipitates with flag-tuberin generally correlates with the amount of flag-tuberin present in the immunoprecipitate (Figure 4B, third panel from top).

We have also used phospho-specific antibodies to T1462 to demonstrate PI3K-dependent phosphorylation of endogenous tuberin. To first test the specificity of the tuberin phospho-T1462 (tuberin-pT1462) antibody, we immunoprecipitated flag-tagged wild-type tuberin and the tuberin<sup>T1462A</sup> mutant from HEK-293 cells. This antibody has high specificity for wild-type tuberin over tuberin<sup>T1462A</sup> (Figure 4C). Furthermore, its recognition of

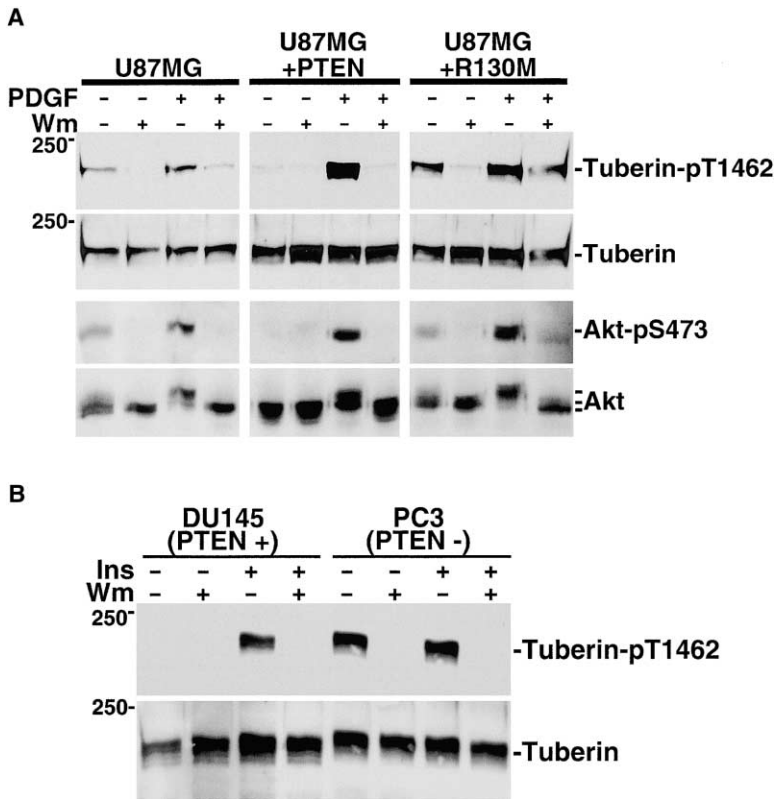


Figure 5. Tuberin Is Constitutively Phosphorylated on T1462 in PTEN<sup>-/-</sup> Tumor-Derived Cell Lines

(A) Growth-factor-independent phosphorylation of tuberin on T1462 in U87MG glioblastoma cells is dependent on the absence of PTEN phosphatase activity. Cell lysates were prepared from serum-starved U87MG cells or U87MG cells reconstituted with either PTEN or PTEN<sup>R130M</sup> that were either left untreated, treated for 15 min with 100 nM wortmannin (Wm), stimulated for 15 min with 10 ng/ml PDGF, or treated for 15 min with 100 nM wortmannin prior to stimulation with PDGF. Proteins were blotted with the tuberin-pT1462 (top) and the Akt-pS473 (third panel from top) antibodies, then, following stripping, were blotted with the tuberin (second panel from top) and Akt (bottom) antibodies.

(B) Growth-factor-independent phosphorylation of tuberin on T1462 in a PTEN<sup>-/-</sup> prostate tumor-derived cell line. Cell lysates were prepared from serum-starved DU145 or PC3 cells that were treated as in (A), except cells were stimulated with 100 nM insulin instead of PDGF. Proteins were blotted with the tuberin-pT1462 antibody (top) and, following stripping, with the tuberin antibody (bottom).

tuberin is inhibited by wortmannin, demonstrating the specificity of the antibody for tuberin phosphorylated on this PI3K-regulated site. The tuberin-pT1462 antibody recognizes endogenous tuberin from NIH-3T3 cell lysates in a growth factor- and PI3K-dependent manner (Figure 4D). Therefore, as determined with both the Akt-pSub antibody and this phospho-tuberin-specific antibody, tuberin is phosphorylated on T1462 upon activation of the PI3K-Akt pathway.

#### Tuberin Is Constitutively Phosphorylated in PTEN<sup>-/-</sup> Tumor-Derived Cell Lines

Absence of the phosphoinositide 3-phosphatase PTEN leads to a large increase in basal levels of PI3K lipid products and in Akt activity (Maehama and Dixon, 1998; Ramaswamy et al., 1999; Sun et al., 1999; reviewed by Cantley and Neel, 1999). Based on the collective data above, we hypothesized that tuberin should be constitutively phosphorylated in cell lines derived from PTEN-negative tumors. We first tested this idea with the PTEN<sup>-/-</sup> glioblastoma cell line U87MG (Myers et al., 1998). Tuberin is phosphorylated on T1462 even in U87MG lysates from cells under serum-starved conditions (Figure 5A). There is a slight increase over basal levels of phosphorylation upon stimulation with PDGF. Under both conditions, tuberin phosphorylation is blocked by inhibition of PI3K with wortmannin. Importantly, this increase in basal phosphorylation of tuberin is dependent on the absence of PTEN from this cell line. In U87MG cells reconstituted with wild-type PTEN but not the phosphatase dead PTEN<sup>R130M</sup> mutant, the growth factor dependence for phosphorylation of tuberin is re-

stored (Figure 5A, top panel). Under all of these conditions, the phosphorylation status of tuberin correlates with activation of Akt (Figure 5A, third row from top).

PTEN mutations are common in prostate cancer (Cairns et al., 1997). We obtained two prostate tumor-derived cell lines, DU145 (PTEN positive) and PC3 (PTEN negative; Whang et al., 1998), in order to access the phosphorylation state of tuberin. Tuberin is not phosphorylated on T1462 in serum-starved DU145 cells, but has high constitutive phosphorylation, which is wortmannin sensitive, in serum-starved PC3 cells (Figure 5B, top panel). Phosphorylation of T1462 is detected in DU145 lysates specifically from cells stimulated with insulin, while no discernable difference in tuberin phosphorylation is detected between serum-starved and insulin-stimulated conditions in PC3 cells (Figure 5B, top panel). Therefore, as seen above with the glioblastoma cell line, tuberin is aberrantly phosphorylated in the absence of growth stimuli when PTEN is mutated, and this is dependent on PI3K activity.

#### A Tuberin Mutant that Cannot Be Phosphorylated on S939 and T1462 Inhibits S6K1 Activation

Recent fruit fly and mouse genetics on the *TSC1* and *TSC2* genes have suggested that S6K1 is downstream of the tuberin-hamartin complex (Kwiatkowski et al., 2002; Potter et al., 2001; Tapon et al., 2001). The gene for *Drosophila* S6K1 is epistatic to *dTSC1* and *dTSC2* in genetic experiments assaying cell size in the *Drosophila* eye disc (Potter et al., 2001; Tapon et al., 2001). Furthermore, mouse embryonic fibroblasts isolated from a *TSC1* knockout mouse have been shown to express

constitutively phosphorylated and activated S6K1 (Kwiatkowski et al., 2002). Together, these studies imply that the tuberin-hamartin complex functions to inhibit S6K1 activation.

We therefore tested if wild-type tuberin or tuberin<sup>S939A/T1462A</sup> had any effect on phosphorylation and activation of S6K1. HEK-293 cells were cotransfected with HA-S6K1 and either vector alone, FLAG-tuberin or FLAG-tuberin<sup>S939A/T1462A</sup>. Under the transfection conditions used here (see Experimental Procedures), the wild-type and mutant tuberin constructs are expressed at levels that are less than 2-fold over endogenous tuberin (data not shown). The phosphorylation and kinase activity of HA-S6K1 immunoprecipitated from these cells was analyzed. Phosphorylation of S6K1 on T389 in response to mitogens correlates with its activation and requires the activity of both PI3K and mTOR (Weng et al., 1998). As predicted, HA-S6K1 is recognized by a pT389 antibody in response to insulin stimulation, and this is blocked by treatment of cells with either wortmannin or rapamycin (Figure 6A, top panel). Coexpression of wild-type tuberin has little effect on the growth factor-induced phosphorylation of T389. However, the tuberin<sup>S939A/T1462A</sup> mutant greatly reduces the insulin-stimulated phosphorylation of S6K1 on T389. Furthermore, expression of the tuberin<sup>S939A/T1462A</sup> mutant but not wild-type tuberin leads to a significant decrease in both the basal and insulin-stimulated kinase activity of S6K1 toward a recombinant GST-S6 substrate (Figure 6A, third panel from top). The tuberin<sup>S939A/T1462A</sup> mutant but not wild-type tuberin also significantly reduces S6K1 activity from cells growing in full serum (Figure 6B, second panel from top). Importantly, expression of the tuberin<sup>S939A/T1462A</sup> mutant at these levels had no effect on phosphorylation of known Akt targets, including exogenously introduced FKHR or endogenous GSK3 (data not shown). Therefore, a tuberin mutant lacking the major PI3K-dependent phosphorylation sites can block growth factor-induced S6K1 activation. This suggests that these phosphorylation sites are normally required for the PI3K-Akt pathway to relieve tuberin-mediated inhibition of S6K1 (see Discussion).

## Discussion

We have described an approach that uses a combination of biochemistry and bioinformatics to identify new targets of the PI3K-Akt pathway. We find that the *tuberous sclerosis complex-2* gene product, tuberin, is phosphorylated on sites blotted by the Akt-pSub antibody, which recognizes the RxRxxpS/T motif, in a growth factor- and PI3K-dependent manner. We also show that Akt can phosphorylate tuberin both in vitro and in vivo and that a dominant-negative mutant of Akt can inhibit phosphorylation of tuberin by the endogenous kinase. The primary sites of PI3K-regulated phosphorylation on tuberin were mapped to S939 and T1462. A phospho-specific antibody to T1462 demonstrates that endogenous tuberin is phosphorylated upon activation of the PI3K-Akt pathway, either by growth factor stimulation or loss of PTEN. Finally, we find that a tuberin mutant lacking the PI3K-dependent phosphorylation sites has the ability to block growth factor-induced activation of S6K1.

## An Approach to Identify Phospho-Protein Components of Signaling Pathways

The approach presented here is also applicable to identifying targets of other specific protein kinases, as well as targets of protein domains that bind to specific phosphorylated motifs (e.g., SH2 domains) for which peptide library data exist (Songyang and Cantley, 1998; Yaffe and Cantley, 2000). Immunoblotting with phospho-motif-specific antibodies to a consensus kinase recognition or protein binding motif, along with a way to genetically or pharmacologically activate and/or inactivate the pathway of interest, allows estimates of the molecular weights of proteins modified in response to pathway activation. Scansite can then be used to search for candidate proteins for these targets in a particular molecular mass range. Furthermore, Scansite can search databases for proteins specific to a given range of isoelectric points. Therefore, data from phospho-motif antibody immunoblots on proteins resolved by 2-dimensional gel electrophoresis can be used in a combined Scansite search for proteins containing the motif of interest, in a specific size and isoelectric point range. This greatly increases the strength of prediction of target identity. We are currently using this approach to identify strong candidates for the five or six PI3K-dependent phosphoproteins grouped between 130 and 170 kDa on the Akt pSub immunoblots (Figure 1A).

## The PI3K-Akt-Tuberin Pathway and Regulation of S6K1

TSC is a common disease affecting an estimated 1 in 6000 individuals and is characterized by the occurrence of widespread benign tumors called hamartomas frequently affecting the brain, skin, kidneys, lungs, eyes, and heart (reviewed by Gomez et al., 1999). In approximately 85% of TSC patients, the disease is caused by loss-of-function mutations in one of two tumor suppressor genes, *TSC1* and *TSC2*, which encode hamartin and tuberin, respectively. These two proteins form a complex (Nellist et al., 1999). Tuberin, which has a region of homology to Rap1 GTPase-activating proteins (GAPs), has been shown to possess in vitro GAP activity toward both Rap1 (Wienecke et al., 1995) and Rab 5 (Xiao et al., 1997). However, the true molecular and cellular functions of the hamartin-tuberin complex have yet to be clearly defined. Furthermore, very little is known about how these tumor suppressor gene products are regulated.

We demonstrate here that tuberin is phosphorylated on S939 and T1462 in response to PI3K activation. Our results are consistent with Akt being the PI3K-dependent tuberin kinase, although we cannot rule out the possibility that other kinases downstream of PI3K with similar substrate specificities, such as an SGK family member (Park et al., 1999), could also phosphorylate tuberin. These findings correlate well with recently published *Drosophila* genetic epistasis analyses of *dPI3K*, *dPKB*, *dTSC1*, and *dTSC2*, which place the tuberin-hamartin complex either downstream of PI3K and Akt or in a parallel pathway (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). The findings presented here demonstrate that the human TSC complex is a direct biochemical target of the PI3K-Akt pathway.



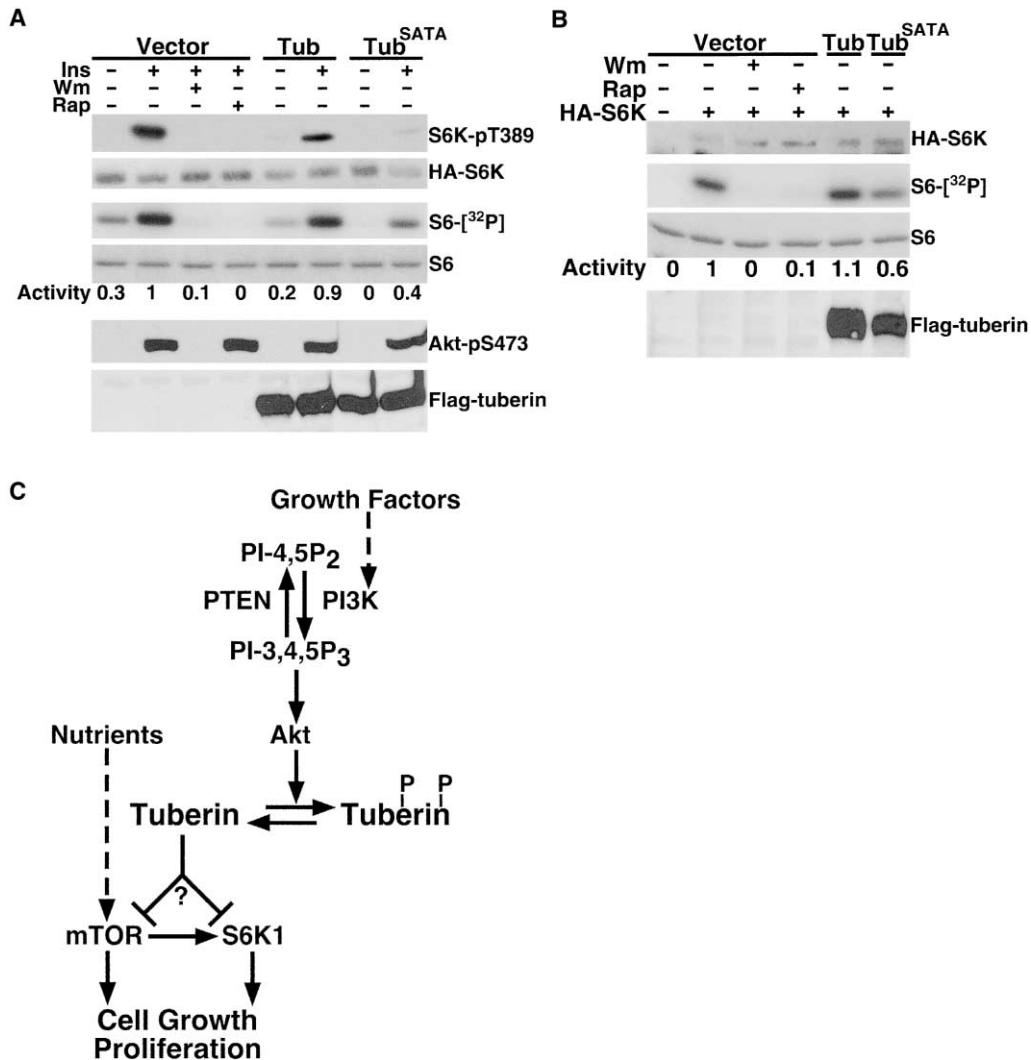


Figure 6. Expression of the Tuberin<sup>S939A/T1462A</sup> Mutant Blocks Growth Factor-Induced S6K1 Activity

(A) The tuberin<sup>S939A/T1462A</sup> mutant inhibits insulin-stimulated S6K1 phosphorylation and activity. In HEK-293 cells, a HA-S6K1 construct was cotransfected with FLAG-vector, FLAG-tuberin, or FLAG-tuberin<sup>S939A/T1462A</sup>. Cell lysates were prepared from cells that were serum starved and left untreated, stimulated with 100 nM insulin for 30 min, or treated with either 100 nM wortmannin or 25 nM rapamycin for 15 min prior to insulin stimulation. Immunoprecipitated HA-S6K1 was blotted with the S6K1-pT389 antibody (top) and the HA antibody (second panel from top), and kinase assays were performed using recombinant GST-S6 as a substrate (third panel from top). The kinase activity was quantitated using a phosphorimager and is shown as the activity relative to immunoprecipitates from insulin-stimulated cells cotransfected with HA-S6K1 and Flag-vector. Proteins from total cell lysates were blotted with the Akt-pS473 (fifth panel from top) and FLAG (bottom) antibodies.

(B) The tuberin<sup>S939A/T1462A</sup> mutant lowers the activity of S6K1 from cells in full serum. HEK-293 cells were transfected as in (A), and cell lysates were prepared from cells in full serum or full serum plus either 100 nM wortmannin (Wm) or 25 nM rapamycin (Rap) for 15 min. Immunoprecipitated HA-S6K1 was blotted with the HA antibody (top), and kinase assays were performed (second panel from top) and activities quantitated as in (A). Proteins from total cell lysates were blotted with the FLAG antibody (bottom). The results shown in (A) and (B) are each representative of at least three independent experiments.

(C) Model of the PI3K-Akt-tuberin pathway and its control of S6K1 activation. Growth and survival factors activate PI3K, which produces the second messenger PI-3,4,5P<sub>3</sub>. The PTEN lipid phosphatase downregulates this signal by dephosphorylating the lipid products of PI3K. Akt binds to PI-3,4,5P<sub>3</sub> and is subsequently activated. Akt then phosphorylates tuberin on S939 and T1462 and thereby inhibits its function via an unknown molecular mechanism. This inhibition of tuberin, in turn, relieves its inhibition of S6K1, and/or mTOR. Two pathways, the PI3K-Akt-tuberin pathway signaling from mitogenic stimuli and the mTOR pathway signaling the availability of nutrients, would therefore converge to activate S6K1.

Based on genetic studies and the fact that *PI3K* and *Akt* are oncogenes while the *TSC* genes are tumor suppressors, one would predict that the PI3K-Akt-mediated phosphorylation of tuberin would inhibit the function of the tuberin-hamartin complex. In *Drosophila*, hamartin and tuberin appear to function together to antagonize

signaling of the insulin-PI3K-Akt pathway and, thereby, restrict cell growth and proliferation (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Most strikingly, loss of just one copy of *TSC1* or *TSC2* partially rescues the lethality of insulin receptor loss-of-function mutants (Gao and Pan, 2001). This result implies that one of

the primary functions of the insulin pathway, at least in *Drosophila*, is to inhibit the hamartin-tuberin complex. Furthermore, both mouse and *Drosophila* genetic studies have suggested that the tuberin-hamartin complex functions to inhibit S6K1 (Kwiatkowski et al., 2002; Potter et al., 2001; Tapon et al., 2001).

We find that expression in human cells of the tuberin<sup>S939A/T1462A</sup> mutant, which lacks the major PI3K-dependent phosphorylation sites, at levels comparable to endogenous tuberin leads to a decrease in growth factor-induced S6K1 phosphorylation and activity. This phosphorylation and subsequent activation of S6K1 has been previously demonstrated to be dependent on PI3K (Chung et al., 1994; reviewed by Martin and Blenis, 2002). These results, along with those from genetic studies in other systems (Kwiatkowski et al., 2002; Potter et al., 2001; Tapon et al., 2001), are consistent with a model in which growth factors activate PI3K leading to the phosphorylation of tuberin by Akt (Figure 6C). This phosphorylation inhibits the tuberin-hamartin complex, thereby relieving its inhibition of S6K1. In this model, expression of the tuberin<sup>S939A/T1462A</sup> mutant, which would not be phosphorylated and inhibited, would have a dominant effect over endogenous tuberin and block growth factor-induced S6K1 activation. It will be of great interest to determine the molecular nature of S6K1 inhibition by the tuberin-hamartin complex in the absence of mitogenic stimuli. It is possible that the complex does so upstream of mTOR, as the constitutive activation of S6K1 in *TSC1*<sup>-/-</sup> MEFs is sensitive to rapamycin (Kwiatkowski et al., 2002). Alternatively, mTOR might regulate S6K1 in a nutrient-sensitive pathway parallel to the mitogen-sensitive PI3K-Akt-tuberin pathway.

Recent studies have suggested that S6K1 activation can occur independent of PI3K and Akt (e.g., Radimerski et al., 2002). We believe that these studies demonstrate the existence of multiple pathways regulating S6K1 and that the tuberin-hamartin complex might integrate signals from many different inputs. The identification of tuberin as a direct downstream target of the PI3K-Akt pathway provides the missing link between this signaling cascade and control of S6K1 activity.

#### Implications for Tuberous Sclerosis Complex and Other Cancer Diseases

The identification of this biochemical relationship between the mammalian *TSC* tumor suppressor gene products and the oncogenic PI3K-Akt pathway could have important implications in human diseases. For instance, in approximately 10%–15% of patients diagnosed with TSC, mutations in *TSC1* or *TSC2* have not been detected (e.g., Dabora et al., 2001). Based on our findings, it is possible that mutations leading to aberrant activation of the PI3K-Akt pathway, such as *PTEN* mutations, could inhibit the function of the tuberin-hamartin complex by causing constitutive phosphorylation of tuberin. It will be interesting to examine the phosphorylation state of tuberin within hamartomas from such TSC patients. Indeed, in *PTEN*<sup>-/-</sup> cell lines derived from both glioblastoma and prostate tumors, we detect growth factor-independent phosphorylation of tuberin on the PI3K-dependent T1462 site.

Germline mutations in either *PTEN* or the *TSC* genes

cause autosomal dominant diseases that are characterized by the occurrence of widespread hamartomas due to loss of heterozygosity at these loci (reviewed by Cantley and Neel, 1999; Gomez et al., 1999). However, the tissue distribution of these benign tumors varies between patients with loss of *PTEN* and those with TSC. These differences might be explained by a model in which the tuberin-hamartin complex is the primary growth-inhibiting target of the PI3K-Akt pathway in tissues affected in TSC patients. In other tissues, such as those affected in patients with *PTEN* mutations, this complex might be one of many targets of the PI3K-Akt pathway. Interestingly, though, recent studies have suggested that mTOR activity is essential for oncogenic transformation of cells by activated PI3K or Akt and for growth of *PTEN*<sup>-/-</sup> tumors (Aoki et al., 2001; Neshat et al., 2001; reviewed by Vogt, 2001). Therefore, aberrant phosphorylation and inhibition of the tuberin-hamartin complex, and subsequent increased activity of mTOR and/or S6K1, would likely contribute to tumorigenesis caused by mutations that activate the PI3K-Akt pathway. Future studies using crosses between *PTEN* and *TSC* knockout mice should help determine the contribution of the tuberin-hamartin complex in prevention of the variety of tumors caused by uncontrolled signaling through the PI3K-Akt pathway. Finally, the elucidation of a PI3K-Akt-tuberin pathway controlling S6K1 activity will have important implications in our understanding and treatment of the prevalent TSC disease.

#### Experimental Procedures

##### Cell Culture and Transfections

All cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The U87MG cell line and its *PTEN*-reconstituted derivatives were a generous gift from the laboratory of N.K. Tonks and have been described previously (Myers et al., 1998). Transfection experiments were carried out with lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) on 60 mm culture dishes according to the manufacturer's instructions (2  $\mu$ g of each plasmid per transfection), and cells were lysed, or first serum-starved overnight, 14–16 hr after transfection. However, for cotransfection experiments testing the ability of tuberin constructs to inhibit S6K1 activity, only 0.5  $\mu$ g of HA-S6K1 and 0.25  $\mu$ g of FLAG-tuberin constructs were used for each transfection.

##### Whole-Cell Lysate Preparation, Immunoprecipitations, and Immunoblotting

Cell lysates were prepared in 1 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1% Nonidet P-40 (NP-40), 10% glycerol, 1 mM dithiothreitol (DTT), 50 mM  $\beta$ -glycerophosphate, 50 mM NaF, 10  $\mu$ g/ml phenylmethylsulfonyl fluoride, 4  $\mu$ g/ml aprotinin, 4  $\mu$ g/ml leupeptin, and 4  $\mu$ g/ml pepstatin [pH 7.4]). For immunoprecipitations, lysates were incubated with the precipitating antibody for 2 hr, followed by a 1 hr incubation with 10  $\mu$ l of protein-A- or protein-G-agarose slurries (Amersham Biosciences Corp., Piscataway, NJ), or for anti-flag immunoprecipitations, lysates were incubated for 2 hr with 10  $\mu$ l of an M2-agarose affinity gel slurry (Sigma-Aldrich Co., St. Louis, MO). Immune complexes on beads were then washed three times in wash buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 50 mM  $\beta$ -glycerophosphate, 50 mM NaF, 10  $\mu$ g/ml phenylmethylsulfonyl fluoride, 4  $\mu$ g/ml aprotinin, 4  $\mu$ g/ml leupeptin, and 4  $\mu$ g/ml pepstatin [pH 7.4]) and boiled in Laemmli sample buffer.

Proteins from lysates (5% of total) and immunoprecipitates were separated by 6%–10% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (PolyScreen,

NEN Life Science Products, Boston, MA). Following blocking, sections of the membrane containing the molecular mass range of proteins of interest were blotted with the appropriate antibody. Most of the antibodies used in the experiments described here were obtained from Cell Signaling Technologies (Beverly, MA) and include the Akt-pSub, Akt-pS473, Akt, GSK-3 $\alpha$ / $\beta$ -pS21/S9, and S6K1-pT389 antibodies. A phospho-threonine-containing peptide of the sequence surrounding T1462 on human tuberin was used by Cell Signaling Technologies to generate the polyclonal tuberin-pT1462 antibody used in this study. Other antibodies used in this study are the tuberin antibody (C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA), HA antibody (HA11; Babco, Richmond, CA), FLAG antibody (M5; Sigma-Aldrich Co.), and a hamartin antibody generously provided by the laboratory of D.J. Kwiatkowski. Following washes, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Chemicon International Inc., Temecula, CA) and detected by enhanced chemiluminescence.

#### Plasmid Constructs

A pcDNA6-TSC2 construct encoding human tuberin was obtained from the laboratory of D.J. Kwiatkowski and was used to generate the FLAG-tuberin-encoding constructs. The sequence encoding the FLAG epitope (MDYKDDDDK) was cloned between the KpnI and BamHI sites on pcDNA3 (Invitrogen) to generate pcDNA3-FLAG (FLAG-vector). The first 276 base pairs of TSC2 were PCR-amplified from pcDNA6-TSC2 and inserted between the BamHI and NotI sites in frame with the FLAG-coding sequence in pcDNA3-FLAG. The remainder of TSC2 was subcloned from pcDNA6-TSC2 and inserted between the NotI and XbaI sites of the above construct. The pcDNA3-FLAG-tuberin<sup>S939A</sup> and pcDNA3-FLAG-tuberin<sup>T1462A</sup> constructs were generated by site-directed PCR mutagenesis. The pcDNA3-FLAG-tuberin<sup>S939A/T1462A</sup> construct was made by inserting the Bsu36I to EcoRV fragment of pcDNA3-FLAG-tuberin<sup>T1462A</sup> into these same sites on pcDNA3-FLAG-tuberin<sup>S939A</sup>. All Akt constructs used in this study were subcloned from pAdTrack CMV-Akt constructs, provided by Toshiyuki Obata, into the pcDNA3.1<sup>+</sup> vector. The pRK7-HA-S6K1 construct was described previously (Romanelli et al., 1999).

#### Kinase Assays

In order to obtain activated Akt for in vitro kinase assays, HEK-293 cells were transfected with HA-Akt or HA-Akt-KD constructs, and cell lysates prepared from these cells in full serum were incubated with HA antibodies for 1 hr. In order to obtain tuberin that is not phosphorylated on sites recognized by the Akt-pSub antibody, HEK-293 cells were transfected with FLAG-vector or FLAG-tuberin constructs, and cell lysates prepared from these cells treated with 200 nM wortmannin for 30 min were incubated with FLAG (M5) antibodies. Half of each HA antibody-containing lysate was then mixed with half of each FLAG antibody-containing lysate, and these mixtures were incubated for 1 hr with protein G-agarose beads. The protein G-agarose bound to immune complexes of both HA-Akt and FLAG-tuberin were then washed three times in wash buffer and two times in kinase reaction buffer (20 mM HEPES, 10 mM MgCl<sub>2</sub>, and 0.5 mM EGTA [pH 7.4]). Kinase reactions were carried out in 30  $\mu$ l kinase reaction buffer with 1 mM DTT, 100 nM ATP, and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]-ATP for 30 min at 37°C, and reactions were stopped by boiling in Laemmli buffer. Proteins from these assays were separated by 6%–10% gradient SDS-PAGE and transferred to a PVDF membrane, and <sup>32</sup>P incorporation into the indicated proteins (Figure 3A) was detected by exposure to film for 2 hr.

The HA-S6K1 immune complex kinase assays were performed as described previously (Chung et al., 1992; Romanelli et al., 1999).

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